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<b>(21) International Application Number:</b> PCT/US98/16766 <b>(22) International Filing Date:</b> 13 August 1998 (13.08.98) <b>(71) Applicant (for all designated States except US):</b> WALTER REED ARMY INSTITUTE OF RESEARCH [US/US]; Department of the Army, Washington, DC 20307 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> ULRICH, Robert, G. [US/US]; 6811 Falstone Drive, Frederick, MD 21702 (US). OLSON, Mark, A. [US/US]; 107 Fairgrove Terrace, Gaithersburg, MD 20877 (US). BAVARI, Sina [US/US]; 109 Fairway Drive, Dillsburg, PA 17019 (US). <b>(74) Agent:</b> HARRIS, Charles, H.; United States Army Medical Research and Material Command, 504 Scott Street, Fort Detrick, MD 21702 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>
<b>(54) Title:</b> BACTERIAL SUPERANTIGEN VACCINES  <b>(57) Abstract</b>  The present invention relates to genetically attenuated superantigen toxin vaccines altered such that superantigen attributes are absent, however the superantigen is effectively recognized and a n appropriate immune response is produced. The attenuated superantigen toxins are shown to protect animals against challenge with wild type toxin. Methods of producing and using the altered superantigen toxins are described.		

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## TITLE OF THE INVENTION

Bacterial Superantigen Vaccines

by

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## INTRODUCTION

10

Staphylococcal enterotoxins (SEs) A through E are the most common cause of food poisoning [Bergdoll, M.S. (1983) In Easom CSF, Aslam C., eds. Staphylococci and staphylococcal infections. London: Academic Press, pp 559-598] and are associated with several serious diseases [Schlievert, P.M. (1993) *J. Infect. Dis.* **167**: 997-1002; Ulrich et al. (1995) *Trends Microbiol.* **3**: 463-468], such as bacterial arthritis [Schwab et al. (1993) *J. Immunol.* **150**: 4151-4159; Goldenberg et al. (1985) *N. Engl. J. Med.* **312**: 764-771], other autoimmune disorders [Psnett, D. N. (1993) *Semin. Immunol.* **5**: 65-72], and toxic shock syndrome [Schlievert, P.M. (1986) *Lancet* **1**: 1149-1150; Bohach et al. (1990) *Crit. Rev. Microbiol.* **17**: 251-272]. The nonenterotoxigenic staphylococcal superantigen toxic shock syndrome toxin-1 (TSST-1) was first identified as a causative agent of menstrual-associated toxic shock syndrome [Schlievert et al. (1981) *J. Infect. Dis.* **143**: 509-516]. Superantigen-producing *Staphylococcus aureus* strains are also linked to Kawasaki syndrome, an inflammatory disease of children [Leung et al. (1993) *Lancet* **342**: 1385-1388].

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The staphylococcal enterotoxins A-E, toxic shock syndrome toxin-1 (TSST-1), and streptococcal pyrogenic

exotoxins A-C are soluble 23-29-kD proteins commonly referred to as bacterial superantigens (SAGs). Bacterial superantigens are ligands for both major histocompatibility complex (MHC) class II molecules, expressed on antigen-presenting cells, and the variable portion of the T cell antigen receptor  $\beta$  chain (TCRV $\beta$ ) [Choi et al. (1989) *Proc. Natl. Acad. Sci. USA* 86:8941-8945; Fraser, J.D. (1989) *Nature* 339:221-223; Marrack et al. (1990) *Science* 248: 705-711; Herman et al. (1991) *Annu. Rev. Immunol.* 9: 745-772; Mollick et al. (1989) *Science* 244:817-820].

Each bacterial superantigen has a distinct affinity to a set of TCRV $\beta$ , and coligation of the MHC class II molecule polyclonally stimulates T cells [White et al. (1989) *Cell* 56: 27-35; Kappler et al. (1989) *Science* 244: 811-813; Takimoto et al. (1990) *Eur. J. Immunol.* 140: 617-621]. Pathologically elevated levels of cytokines that are produced by activated T cells are the probable cause of toxic shock symptoms [Calson et al. (1985) *Cell. Immunol.* 96: 175-183; Stiles et al. (1993) *Infect. Immun.* 61: 5333-5338]. In addition, susceptibility to lethal gram-negative endotoxin shock is enhanced by several bacterial superantigens [Stiles, et al., *supra*]. Although antibodies reactive with superantigens are present at low levels in human sera [Takei et al. (1993) *J. Clin. Invest.* 91: 602-607], boosting antibody titers by specific immunization may be efficacious for patients at risk for toxic shock syndrome and the other disorders of common etiology.

A vaccine approach to controlling bacterial superantigen-associated diseases presents a unique set of challenges. Acute exposure to bacterial superantigens produces T cell anergy, a state of

specific non-responsiveness [Kawabe et al. (1991) *Nature* 349: 245-248], yet T cell help is presumably a requirement for mounting an antibody response.

Presently, the only superantigen vaccines  
5 available are chemically inactivated toxoids from  
different bacteria which have several disadvantages.  
The chemical inactivation process can be variable for  
each production lot making the product difficult to  
characterize. The chemical used for inactivation,  
10 (e.g. formaldehyde), is often toxic and does not  
negate the possibility of reversion of the inactivated  
superantigen to an active form. In addition, the  
yields of wild-type toxin from bacterial strains used  
for making toxoids are often low.

15

#### SUMMARY OF THE INVENTION

The present invention relates to a vaccine which  
overcomes the disadvantages of the chemically  
inactivated toxoids described above. The superantigen  
20 vaccine(s) of the present invention is/are designed to  
protect individuals against the pathologies resulting  
from exposure to one or several related staphylococcal  
and streptococcal toxins. The superantigen vaccine is  
comprised of a purified protein product that is  
25 genetically attenuated by DNA methodologies such that  
superantigen attributes are absent, however the  
superantigen is effectively recognized by the immune  
system and an appropriate antibody response is  
produced.

30 Specifically, the vaccine of the present  
invention is a product of site-directed mutagenesis of  
the DNA coding sequences of superantigen toxins  
resulting in a disruption of binding to both the MHC  
class II receptor and to the T-cell antigen receptor.  
35 A comprehensive study of the relationships of the

superantigen structures of TSST-1, streptococcal pyrogenic exotoxin-A (SPEa), staphylococcal enterotoxin B (SEB), and staphylococcal enterotoxin A, to receptor binding were undertaken to provide insight into the design of the vaccine. From these studies, critical amino acid residues of the toxin responsible for binding the superantigen to the human MHC receptor were defined. Site-directed mutagenesis of the gene encoding the toxin and expression of the new protein product resulted in a superantigen toxin with disrupted binding to the MHC receptors.

Therefore, it is an object of the present invention to provide a superantigen toxin DNA fragment which has been genetically altered such that binding of the encoded altered toxin to the MHC class II or T-cell antigen receptor is disrupted. Such a DNA fragment is useful in the production of a vaccine against superantigen toxin infections.

It is another object of the present invention to provide a superantigen toxin amino acid sequence which has been altered such that the binding of the encoded altered toxin to the MHC class II or T-cell antigen receptor is disrupted. Such a sequence is useful for the production of a superantigen toxin vaccine.

It is another object of the invention to provide a recombinant vector comprising a vector and the DNA fragment described above.

It is a further object of the present invention to provide host cells transformed with the above-described recombinant DNA constructs. Host cells include cells of other prokaryotic species or eukaryotic plant or animal species, including yeasts,

fungi, plant culture, mammalian and nonmammalian cell lines, insect cells and transgenic plants or animals.

It is another object of the present invention to provide a method for producing altered superantigen toxin with disrupted MHC class II and T-cell antigen receptor binding which comprises culturing a host cell under conditions such that a recombinant vector comprising a vector and the DNA fragment described above is expressed and altered superantigen toxin is thereby produced, and isolating superantigen toxin for use as a vaccine against superantigen toxin-associated bacterial infections and as a diagnostic reagent.

It is still another object of the invention to provide a purified altered superantigen toxin useful as a vaccine and as a diagnostic agent.

It is another object of the invention to provide a purified altered superantigen toxin for the therapeutic stimulation of, or other *in vivo* manipulation of, selective T cell subsets, or *ex vivo* manipulation of T cells for *in vivo* therapeutic purposes in mammals. Diseases, such as autoimmunity, wherein T-cell responses of limited diversity (oligoclonal) are evident. Altered superantigens may be used to therapeutically inactivate (induce anergy in) T cells in diseases wherein oligoclonal T-cell responses are evident such as autoimmune diseases, for example. For diseases in which specific T-cell subsets are not active or are anergic, altered superantigens may be used to therapeutically stimulate these T cells. Such diseases include, but are not limited to, infectious diseases and cancers wherein specific subsets of cytotoxic or helper T cells are inactivated or are otherwise unable to respond normally to the antigenic stimulation of the disease moiety.

It is a further object of the present invention to provide an antibody to the above-described altered superantigen toxin for use as a therapeutic agent and as a diagnostic agent.

5 It is yet another object of the invention to provide a superantigen toxin vaccine comprising an altered superantigen toxin effective for the production of antigenic and immunogenic response resulting in the protection of an animal against  
10 superantigen toxin infection.

It is a further object of the invention to provide a multivalent superantigen toxin vaccine comprising altered toxins from a variety of streptococcal and staphylococcal toxins effective for  
15 the production of antigenic and immunogenic response resulting in the protection of an animal against infection with bacterial superantigen toxin-expressing strains and against other direct or indirect exposures to bacterial superantigen toxins such as might occur  
20 by ingestion, inhalation, injection, transdermal or other means.

It is yet another object of the present invention to provide a method for the diagnosis of superantigen toxin-associated bacterial infection comprising the  
25 steps of:

(i) contacting a sample from an individual suspected of having a superantigen toxin-associated bacterial infection with antibodies which recognize superantigen toxin using antibodies generated from the  
30 altered superantigen toxin; and

(ii) detecting the presence or absence of a superantigen-associated bacterial infection by detecting the presence or absence of a complex formed between superantigen toxin in said sample and  
35 antibodies specific therefor.



It is yet another object of the present invention to provide a method for the diagnosis of superantigen bacterial infection comprising the steps of:

- 1 (i) contacting a sample from an individual  
5 suspected of having the disease with lymphocytes which recognize superantigen toxin produced by said superantigen bacteria or lymphocytes which recognize altered superantigen toxin; and
- 10 (ii) detecting the presence or absence of responses of lymphocytes resulting from recognition of superantigen toxin. Responses can be, for example, measured cytokine release, increase of activation markers, mitotic activity, or cell lysis. The lymphocytes responding to the altered superantigen  
15 toxins recognize them as recall antigens not as superantigens, therefore the response is an indicator of prior exposure to the specific superantigen. The absence of a response may indicate no prior exposure, a defective immune response or in some cases a  
20 manifestation of T-cell anergy. Anergy is defined here as antigen-specific or a generalized non-responsiveness of subsets of T cells.

It is a further object of the present invention to provide a diagnostic kit comprising an antibody  
25 against an altered superantigen toxin and ancillary reagents suitable for use in detecting the presence of superantigen toxin in animal tissue or serum.

It is another object of the present invention to provide a detection method for detecting superantigen  
30 toxins or antibodies to superantigen toxin in samples, said method comprising employing a biosensor approach. Such methods are known in the art and are described for example in Karlsson et al. (1991) *J. Immunol. Methods* **145**, 229-240 and Jonsson et al. (1991)  
35 *Biotechniques* **11**, 620-627.

It is yet another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of superantigen-associated bacterial infection, said method comprising providing to an individual in need of such treatment an effective amount of sera from individuals immunized with one or more altered superantigen toxins from different bacteria in a pharmaceutically acceptable excipient.

It is further another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of superantigen toxin-associated bacterial infection, said method comprising providing to an individual in need of such treatment an effective amount of antibodies against altered superantigen toxins in a pharmaceutically acceptable excipient.

It is another object of the present invention to provide a therapeutic method for the treatment or amelioration of symptoms of bacterial superantigen toxin infection, said method comprising providing to an individual in need of such treatment an effective amount of altered superantigen from a variety of streptococcal and staphylococcal bacteria in order to inhibit adhesion of superantigen bacterial toxin to MHC class II or T-cell receptors by competitive inhibition of these interactions.

It is yet another object of the present invention to provide a therapeutic method for the treatment of diseases that may not be associated directly with superantigen toxins but which result in specific nonresponsiveness of T-cell subsets, said method comprising the administration of altered superantigen toxins, *in vivo* or *ex vivo*, such that T-cell subsets are expanded or stimulated. Diseases which cause

anergy or nonresponsiveness of T-cells include, but are not limited to, infectious diseases.

It is another object of the present invention to provide a therapeutic method for the treatment of diseases associated with expanded or over-stimulated T-cell subsets, such as autoimmunity for example, said method comprising administration of altered superantigen toxin, *in vivo* or *ex vivo*, such that anergy or inactivation of disease associated T-cells is produced. In this case, superantigen mutants can be designed with altered but not attenuated T-cell receptor binding, to cause anergy of only the select (i.e. 1-3) T-cell subsets that are pathologically activated.

#### BRIEF DESCRIPTION OF THE DRAWINGS

These and other features, aspects, and advantages of the present invention will become better understood with reference to the following description and appended claims, and accompanying drawings where:

**Figure 1.** Staphylococcal and streptococcal superantigen amino acid sequence homologies, compiled with Genetics Computers Group of Univ. of Wisconsin software.

**Figure 2.** Comparison of mutant SEB and SEA biological activities.

**A.** SEB mutant HLA-DR1-binding; **B.** SEA mutant HLA-DR1-binding; **C.** T-cell recognition of SEA and SEB mutants. Binding of bacterial superantigens to cell surface DR1 was measured by laser fluorescence-activated flow cytometry. A representative experiment of three performed is shown. The mutants SEA D197N, the homologous SEB D199N, and SEA L11Y had no effect on binding or T-cell recognition, and were used for

controls. Human T-cell proliferation, assessed by [<sup>3</sup>H]thymidine incorporation, was measured in response to SEA (Y64A) or SEB (Y61A) mutants and controls that retained DR1-binding affinities. Each data point  
5 represents the mean of triplicate determinations; SEM <5%.

**Figure 3.** Sequence and secondary structural alignment of bacterial superantigen toxins. Analyses were performed with the applications PILEUP and  
10 PROFILE from the Computer Genetics Group (Madison, WI) using sequence data obtained from a variety of sources. Amino acid residue numbering is based on the SEA sequence.

**Figure 4.** Detection of TNF- $\alpha$  (a), IL-1 $\alpha$  (B), IL-  
15 6 (C) and IFN- $\gamma$  (D) in the serum of mice injected with SEA (open circles), LPS (open triangles), or SEA plus LPS (open squares). Values for TNF- $\alpha$  and IL-1 $\alpha$  represent the mean of duplicate samples, with an SEM of  $\pm$  5%. INF- $\gamma$  and IL-6 values represent the mean of  
20 duplicate and triplicate samples, respectively. The SEMs for IFN- $\gamma$  and IL-6 readings were  $\pm$  5% and  $\pm$  10%, respectively.

**Figure 5.** Mutant SEA vaccines that have attenuated major histocompatibility complex class II  
25 or T-cell antigen receptor binding do not induce T-cell anergy. Mice were given three doses of wild type (WT) SEA or site-specific mutant vaccine, plus adjuvant. Control animals received adjuvant alone or were untreated; 2 weeks after final injection, pooled  
30 mononuclear cells were collected from spleens of 4 mice from each group. Results are represented as mean cpm ( $\pm$ SD) of quadruplicate wells incubated with 100 ng/ml WT SEA for 72 h and then pulse-labeled for 12 h with [<sup>3</sup>H]thymidine.  $P < 0.0001$  (analysis of variance

for repeated measures comparing untreated, adjuvant, Y64A, and Y92A to WT SEA group).

**Figure 6.** No superantigen-induced T-cell anergy is exhibited by rhesus monkeys immunized with the vaccine B899445. Peripheral blood lymphocytes were incubated with titrated concentrations of wild-type superantigens from individual rhesus monkeys (K422 and N103) that were immunized with B899445. T-cell proliferation was assessed by [<sup>3</sup>H]thymidine incorporation. Each data point represents the mean of triplicate determinations; SEM <5%.

**Figure 7.** Antibody responses of rhesus monkeys immunized with a combined vaccine consisting of B899445 (SEB) and A489270 (SEA). The antibody levels were measured by ELISA, using plates coated with SEA, SEB or SEC1 as listed. Monkey G8 is a non-immunized control. SEM <5% for triplicate measurements.

#### DETAILED DESCRIPTION

The present invention relates in part to a vaccine against superantigen toxin-associated bacterial diseases. The superantigen vaccines used in this study were developed by engineering changes in the receptor-binding portions of superantigen toxins to reduce receptor-binding affinities and toxicity while maintaining antigenicity.

Five different superantigen vaccines are described in this application: staphylococcal enterotoxin A, staphylococcal enterotoxin B, staphylococcal enterotoxin C1, toxic-shock syndrome toxin-1, and streptococcal pyrogenic exotoxin-A. For each of the superantigen toxins above, a comprehensive study of the relationships of the toxin protein structure to receptor binding was undertaken to provide insight into the design of the vaccine. The

study employed site-directed mutagenesis of toxin and receptor molecules, molecular modeling, protein structure and binding studies. Following these studies, toxins were altered by site-directed  
5 mutagenesis to attenuate MHC class II binding and biological activity to an essentially non-specific level. The engineered vaccines were evaluated at each stage of analysis to determine mouse and human T-cell reactivities *in vitro*, serological responses and  
10 toxicity in mice and monkeys.

In one embodiment, the present invention relates to an altered superantigen toxin protein having an amino acid sequence which has been altered such that the binding of the toxin to the MHC class II receptor  
15 is disrupted.

Comparison of amino acid sequences (Fig. 1) suggested that bacterial superantigens fall into groups consisting of (1) SEA, SED and SEE, (2) SEB, staphylococcal enterotoxins C1-C3 (SEC1-3), the  
20 streptococcal pyrogenic exotoxins A (SPE-A) and C (SPE-C), (3) TSST-1 and (4) the exfoliative toxins (ETA, ETB) and streptococcal pyrogenic exotoxin B (SPE-B), which are the most distant from the others in sequence. Although not available to the inventor when  
25 the inventions were first conceived and proof of principle was obtained, the x-ray crystallographic structures of several bacterial superantigens are now known. Diverse superantigens, such as SEB and TSST-1, appear to have little sequence in common, yet they  
30 exhibit homologous protein folds composed largely of  $\beta$  strands [Prasad, G.S. et al. (1993) *Biochemistry* 32, 13761-13766; Acharya, R.K. et al. (1994) *Nature* 367, 94-97; Swaminathan, S. et al. (1992) *Nature* 359, 801-806] within two distinct domains. Differences between  
35 the proteins are located primarily in highly variable

regions comprised of several surface loops, such as the disulfide-bonded loop which is absent from TSST-1 and at the amino terminus.

5 The X-ray crystal structures of SEB and TSST-1 complexed with HLA DR1 are known [Kim, J. et al. (1994) *Science* **266**, 1870-1874 ; Jardetzky, T.S. et al. (1994) *Nature* **368**, 711-718]. The region of HLA DR1 that contacts SEB consists exclusively of  $\alpha$  subunit surfaces. The main regions of SEB involved are two  
10 conserved sites: a polar pocket derived from three  $\beta$  strands of the  $\beta$  barrel domain and a highly solvent-exposed hydrophobic reverse turn. The polar binding pocket of SEB contains a glutamate and two tyrosines that accommodate Lys39 of the  $\alpha$  subunit of HLA DR1,  
15 while the hydrophobic region consists of a leucine and flanking residues that make several contacts with the HLA DR $\alpha$  chain. The HLA DR1 binding sites of both TSST-1 and SEB overlap significantly. The hydrophobic binding contacts of other SAg with the HLA DR $\alpha$  chain  
20 have been proposed [Ulrich, et al. (1995). *Nature, Struct. Biol* **2**, 554-560] to be similar to those found in SEB and TSST-1. A motif consisting of a leucine in a reverse turn [Ulrich et al. (1995), *supra*] is conserved among bacterial superantigens and may  
25 provide the key determinant (hydrophobic or otherwise) for binding HLA-DR. However, TSST-1 does not have a highly charged residue in the polar pocket that interacts with Lys39 of the HLA DR $\alpha$  chain and uses an alternative conformational binding mode that allows  
30 TSST-1 to interact with HLA DR1  $\beta$ -chain residues and the carboxy-terminal region of the antigenic peptide.

Both SEA and SEE bind to the  $\beta$  subunit of DR by means of a single zinc atom [Fraser, J.D. et al. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 5507-5511]. The  
35 amino-terminal domain of SEA interfaces with the HLA

DR $\alpha$  chain [Ulrich, et al. (1995)], while SEA C-terminal domain residues His187, His225 and Asp227 form a zinc-coordination complex, likely with His-81 from the  $\beta$  chain of an adjoining HLA DR molecule.

5 However, our results have shown that binding of superantigen to the HLA DR $\beta$  subunit does not directly stimulate T cells [Ulrich et al. (1995) *Nature, Struct. Biol.* 2, 554-560], but increases the potential of the bound SEA to interact with the  $\alpha$  chain of  
10 another HLA DR, thus increasing the biological potency.

A least-squares superimposition of the unbound molecules of modeled SEA and the crystal structure of SEB, aligned according to their structurally conserved  
15  $\alpha$ -helical and  $\beta$ -strand regions, exhibited a global folding pattern which is very similar. Differences between the two structures are calculated to be located primarily in loops of low sequence homologies, with the largest positional deviations occurring  
20 between structurally conserved regions of residues 18-20, 30-32, 173-181, 191-194, and the cysteine-loop region (90-111). Only one of these regions in SEB makes significant contact (residue Y94 [Y=tyrosine] in particular) with the HLA-DR1 molecule [Jardetzky, T.S.  
25 et al. (1994) *Nature* 368, 711-718].

The binding interface between SEB and HLA-DR1 consists principally of two structurally conserved surfaces located in the N-terminal domain: a polar binding pocket derived from three  $\beta$ -strand elements of  
30 the  $\beta$ -barrel domain and a hydrophobic reverse turn. The binding pocket of SEB contains residues E67 (E=Glutamic acid), Y89 (Y=Tyrosine) and Y115 (Y=tyrosine), and binds K39 (K=Lysine) of the DR $\alpha$  subunit. The amino acid one letter code is defined as  
35 the following: A= Alanine (Ala), I= Isoleucine (Ile),



L= Leucine (Leu), M= Methionine (Met), F= Phenylalanine (Phe), P= Proline (Pro), W=Tryptophan (Trp), V=Valine (Val), N= Asparagine (Asn), C=Cysteine (Cys), Q= Glutamine (Q), G= Glycine (Gly), S= Serine (Ser), T= Threonine (Thr), Y= Tyrosine (Tyr), R= Arginine (Arg), H=Histidine (His), K= Lysine (Lys), D= Aspartic acid (Asp), and E= Glutamic acid (Glu). For SEA, the binding interface with the DR molecule is modeled to contain a similar binding pocket consisting of residues D70, Y92 and Y108. Mutation of residue Y89 in SEB or Y92 in SEA to alanine (**Fig. 2**) resulted in greater than 100-fold reduction in DR1 binding. The substitution of alanine for Y89 in SEB and Y92 in SEA eliminates the hydrogen bond with K39 and disrupts packing interactions with adjacent protein residues. Modeling of the SEA mutant Y92A predicts an increase in solvent-accessible surface area for Y108 by a factor of two greater than the wild-type structure, allowing the formation of a hydrogen bond to the carboxylate group of D70 and thus disrupting key anchoring and recognition points for HLA-DR1. This effect is expected to be somewhat less in SEB due to the longer side chain at E67. Substitution of SEB Y115 with alanine also resulted in greater than 100-fold reduction of binding. In contrast, the same replacement of Y108 in SEA yielded little to no change in DR1 binding (**Fig. 2a**), suggesting the primary importance of SEA residues Y92 and D70 for stabilizing interactions with K39. The K39 side chain of DR $\alpha$  forms a strong ion-pair interaction with the SEB E67 carboxylate group and hydrogen bonds with the hydroxyl groups of Y89 and Y115. Substitution of SEB E67 by glutamine reduced binding affinity by greater than 100-fold (**Fig. 2**), reflecting the replacement of the strong ionic bond with a weaker hydrogen bond. To

optimize ion-pair interactions of the analogous SEA site, the shorter carboxylate side chain of D70 is predicted to shift K39 of DR $\alpha$ , weakening interactions with SEA Y108. The substitution of alanine for SEA  
5 Y108 is thus more easily accommodated than the homologous substitution of SEB Y115, without loss in DR1 binding.

Comparisons of the polar pocket with other bacterial superantigens were then made. SEC1-3 and  
10 SPE-A have conserved the critical DR1 binding-interface residues (**Fig. 1**), and share with SEB and SEA secondary structural elements of the DR1-binding surfaces. Asparagine in SED (N70) replaces the acidic side chain present in SEA, SEB, SPE-A and SEC1-3.  
15 Accordingly, for SED the salt bridge of the polar pocket is likely to be replaced by a hydrogen bond. Overall, DR1 affinities for SED and SEA appeared to be equivalent (**Fig. 2b**), indicating that other interactions may compensate for the absence in SED of  
20 the ion-pair found in the other superantigens. For the case of TSST-1, mutating DR $\alpha$  residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding [Panina-Bordignon et al. (1992) *J. Exp. Med.* **176**: 1779-1784]. Although primarily  
25 hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB residues Y89 and Y115 are homologous to T69 and I85 in TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned  
30 in a conserved  $\beta$ -barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue T69 would require that DR $\alpha$  K39 extend 5 Å into the pocket. TSST-1 binding  
35 utilizes an alternative strategy [Kim et al. (1994)]

Science 266:1870-1874] consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DR $\alpha$  residues E71 and K67 to R34 and D27, respectively, of TSST-1.

5       The hydrophobic region of the binding interface between SEB and the HLA-DR1 molecule consists of SEB residues 44-47, located in a large reverse turn connecting  $\beta$ -strands 1 and 2 of SEB. These residues appear to make strong electrostatic interactions with  
10 DR $\alpha$  through their backbone atoms. The mutation of L45 to an arginine reduced overall HLA-DR1 binding greater than 100-fold (**Fig. 2b**), attributable to the less energetically favorable insertion of a highly charged residue into a hydrophobic depression on the DR1  
15 molecule. The modeled DR1-SEA complex presents similar interactions with the SEA backbone atoms, with the exception of a glutamine (Q49) replacing SEB Y46. Mutation of L48 to glycine in SEA (homologous to L45 of SEB) has been reported to decrease T-cell  
20 responses. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface. The leucine is conserved among the bacterial superantigens (**Fig. 3**) and may provide the necessary hydrophobic structural element for surface  
25 complementarity with DR1, consistent with the mutagenesis data for SEB and SEA.

The inventor has performed similar structure and function studies with TSST-1, SEC1 and SPE-A.

30       In determining the overall affinity of the superantigen for DR1, a contributory role is played by structural variations around the common binding motifs. A short, variable structured, disulfide-bonded loop is found in SEA and a homologous longer loop in SEB. The SEB residue Y94, contained within  
35 this loop, forms hydrophobic interactions with L60 and

A61 of the DR $\alpha$  subunit. Replacement of Y94 with alanine partially inhibits DR1 binding (Fig. 2a,b). An alanine is found in SEA (A97) and SEE at the position equivalent to SEB Y94, and mutating this residue in SEA to tyrosine results in disrupted instead of stabilized interactions with DR1 (Fig. 2a). Although the disulfide loops differ in structure between SEA and SEB, A97 apparently contributes to the DR $\alpha$  binding interface in a manner similar to Y94 of SEB. Because TSST-1 lacks a disulfide loop, similar contacts with DR $\alpha$  are replaced by interactions with  $\beta$ -strands of TSST-1. In a like manner, the absence of a salt bridge between the residues K39 of DR $\alpha$  and N65 of SED is apparently compensated for by stabilizing interactions occurring outside of the otherwise conserved dominant binding surfaces (Fig. 2a).

The amino acid residues in contact with TCR are located in regions of high sequence variability, presenting a unique surface for interaction with the TCR. Residues implicated in TCR interactions by mutagenesis of SEA and SEB reside in variable loop regions, while TSST-1 mutants that affect TCR binding are mainly located in an  $\alpha$  helix [Acharya, R.K. et al. (1994) *Nature* 367, 94-97; Kim, J. et al. (1994) *Science* 266, 1870-1874]. Specifically, mutations that diminish T-cell receptor recognition of SEB include residues N23, Y61, and the homologous SEA N25 or Y64 (Fig. 2c). SEA residues S206 and N207 also control T-cell responses [Hudson, et al. (1992) *J. Exp. Med.* 177: 175-184]. Mutants of the polar binding pocket, SEA Y92A and SEB Y89A, equivalently reduced T-cell responses (Fig. 2c), reflecting the observed decreases in DR1-binding (Fig. 2a, b). While supporting reduced T-cell responses, mutants SEA Y64A

and SEB Y61A retained normal affinities for DR1 (Fig. 2a-c).

In view of the detailed description of the present invention and the results of molecular modelling and structural studies of staphylococcal and streptococcal superantigen toxins discussed above, any amino acid sequence derived from a superantigen toxin can be altered. Sequences of several superantigen toxins are already known and available to the public in sequence databases such as GenBank, for example. The superantigen toxin sequence is preferably altered at the hydrophobic loop or polar binding pocket depending on the superantigen. Alternatively, residues adjacent to the hydrophobic loop or polar binding pocket that contact HLA-DR or residues at sites that can indirectly alter the structure of the hydrophobic loop or polar pocket can be altered. The number of residues which can be altered can vary, preferably the number can be 1-2, more preferably 2-3, and most preferably 3-4, or more with the limitation being the ability to analyze by computational methods the consequences of introducing such mutations. The residues which can be altered can be within 5 amino acid residues of the central Leucine of the hydrophobic loop (such as L45 of SEB), or within 5 residues of one of the amino acid residues of the polar binding pocket that can contact HLA-DR, (such as E67, Y89, or Y115 of SEB), more preferably, within 3 amino acid residues of the central Leucine of the hydrophobic loop (such as L45 of SEB), or within 3 residues of one of the amino acid residues of the polar pocket that can contact HLA-DR, (such as E67, Y89, or Y115 of SEB), and most preferably, the central Leucine of the hydrophobic loop (such as L45 of SEB), or one of the amino acid residues of the polar binding

pocket that can contact HLA-DR, (such as E67, Y89, or Y115 of SEB). The residues can be changed or substituted to alanine for minimal disruption of protein structure, more preferably to a residue of opposite chemical characteristics, such as hydrophobic to hydrophilic, acidic to neutral amide, most preferably by introduction of a residue with a large hydrated side chain such as Arginine or Lysine. In addition, side chains of certain nonconserved receptor-binding surfaces, can also be altered when designing superantigen toxins with low binding affinities. These residues can include Y94 of SEB and structurally equivalent residues of other superantigens, such as A97 of SEA, or any side chain within 5 residues from these positions or any side chain in discontinuous positions (discontinuous positions are defined as amino acid residues that fold together to form part of a discrete three-dimensional structural unit but are not present on the same secondary structural unit e.g.  $\alpha$  helix or  $\beta$ -strand) such as disulfide-bonded side chains, that involve, directly or indirectly, the nonconserved receptor contact surfaces outside of the polar binding pocket or hydrophobic loop. Further, amino acid residues involved with protein folding or packing can be altered when designing superantigen toxins with low binding affinities [Sundstrom et al. (1996) *EMBO J.* **15**, 6832-6840; Sundstrom et al. (1996) *J. Biol. Chem.* **271**, 32212-32216; Acharya et al. (1994) *Nature* **367**, 94-97; Prasad et al. (1993) *Biochem.* **32**, 13761-13766; Swaminathan et al. (1992) *Nature* **359**, 801-806]. Furthermore, especially for superantigens with higher affinities for T-cell antigen receptors, side chains of amino acids within 5 residues of the position represented by N23 (conserved residue in most

superantigens) , N60 (conserved Asn or Trp in most superantigens) Y91 (semiconserved hydrophobic residues Trp, Ile, Val, His in most superantigens) and D210 of SEB (conserved Asp in most superantigens) can be  
5 altered when designing superantigen toxins with low binding affinities. These residues are likely to form part of the integral molecular surfaces that are in contact with T-cell antigen receptors. Because the T-cell receptor contact areas of superantigen toxins are  
10 essential for causing specific activation or inactivation of T-cell subsets, altering residues that are unique to each superantigen but that are located within 5 residues of the positions represented by N23, N60 and Y91 can produce superantigens that affect a  
15 smaller number (e.g. 1-3) of subsets. Such altered superantigen toxins can be useful as therapeutic agents.

In another embodiment, the present invention relates to a DNA or cDNA segment which encodes a  
20 superantigen toxin such as SEA, SEB, SEC-1, SPEa, and TSST-1 to name a few, the sequence of which has been altered as described above to produce a toxin protein with altered binding ability to MHC Class II and/or T-cell receptors. For SEA, the following three  
25 mutations were introduced into the toxin molecule: Tyrosine at amino acid position 92 changed to alanine; Aspartic acid at amino acid position 70 changed to arginine; Leucine at amino acid position 48 changed to arginine. The reduction in binding to HLA DR is  
30 additive per mutation, though one or two mutations can produce a vaccine and a combination of all three mutations in one molecule produces a better vaccine. Other substitutions can also result in reduced binding.

The B899445 vaccine consists of the following three mutations simultaneously introduced into the toxin molecule: tyrosine at amino acid position 89 changed to alanine; tyrosine at amino acid position 94  
5 changed to alanine; leucine at amino acid position 45 changed to arginine. The altered superantigen toxins can be expressed either as a full-length propolypeptide or as a polypeptide in which the leader peptide has been deleted. The full-length expressed  
10 product (SEA vaccine, A489270P; SEB vaccine B899445P, B2360210P) is secreted into the periplasmic space of *E. coli* host cells, and the leader peptide is recognized and cleaved by a native bacterial enzymatic mechanism. The altered superantigen toxins in which  
15 the leader peptide has been deleted (A489270C, B899445C), the first residue of the mature protein is encoded by the transcriptional start site and codon for methionine (ATG), and the protein is expressed as a nonsecreted product within the host *E. coli* cell.  
20 For the TSST-1 vaccine TST30, the leucine at position 30 was changed to arginine. For the SEC1 vaccine, SEC45, the leucine at position 45 was changed to arginine. For the SPE-A vaccine, SPEA42, the leucine at position 42 was changed to arginine.  
25 In another embodiment, the present invention relates to a recombinant DNA molecule that includes a vector and a DNA sequence as described above. The vector can take the form of a plasmid such as any broad host range expression vector for example  
30 pUC18/19, pSE380, pHIL, pET21/24 and others known in the art. The DNA sequence is preferably functionally linked to a promoter such that the gene is expressed when present in an expression system and an altered superantigen toxin is produced. The expression system  
35 can be an *in vitro* expression system or host cells



such as prokaryotic cells, or *in vivo* such as DNA vaccines.

In a further embodiment, the present invention relates to host cells stably or transiently transformed or transfected with the above-described recombinant DNA constructs. The host can be any eukaryotic or prokaryotic cell including but not limited in *E. coli* DH5 $\alpha$  or BL21. The vector containing the altered superantigen toxin gene is expressed in the host cell and the product of the altered toxin gene, whether a secreted mature protein or a cytoplasmic product, can be used as a vaccine or as a reagent in diagnostic assays or detection methods, or for therapeutic purposes. Please see e.g., Maniatis, Fitch and Sambrook, Molecular Cloning: A Laboratory Manual (1982) or DNA Cloning, Volumes I and II (D. N. Glover ed. 1985) for general cloning methods. The DNA sequence can be present in the vector operably linked to a highly purified IgG molecule, an adjuvant, a carrier, or an agent for aid in purification of altered toxin. The transformed or transfected host cells can be used as a source of DNA sequences described above. When the recombinant molecule takes the form of an expression system, the transformed or transfected cells can be used as a source of the altered toxin described above.

A recombinant or derived altered superantigen toxin is not necessarily translated from a designated nucleic acid sequence; it may be generated in any manner, including for example, chemical synthesis, or expression of a recombinant expression system. In addition the altered toxin can be fused to other proteins or polypeptides for directing transport for example into the periplasm or for secretion from the

cell. This includes fusion of the recombinant or derived altered superantigen to other vaccines or sequences designed to aid in purification, such as His-tagged, epitope-tagged or antibody Fc-fusions.

5 In a further embodiment, the present invention relates to a method of producing altered superantigen toxin which includes culturing the above-described host cells, under conditions such that the DNA  
10 fragment is expressed and a superantigen toxin protein is produced. The superantigen toxin can then be isolated and purified using methodology well known in the art such as immunoaffinity chromatography or preparative isoelectric focusing. However, the method  
15 of purification is not critical to the performance of the vaccine. The altered superantigen toxin can be used as a vaccine for immunity against infection with bacterial superantigen toxins or as a diagnostic tool for detection of superantigen toxin-associated disease or bacterial infection. The transformed host cells  
20 can be used to analyze the effectiveness of drugs and agents which affect the binding of superantigens to MHC class II or T-cell antigen receptors. Chemically derived agents, host proteins or other proteins which result in the down-regulation or alteration of  
25 expression of superantigen toxins or affect the binding affinity of superantigen toxins to their receptors can be detected and analyzed. A method for testing the effectiveness of a drug or agent capable of altering the binding of superantigen toxins to  
30 their receptors can be for example computer-aided rational design or combinatorial library screening, such as phage display technology.

In another embodiment, the present invention relates to antibodies specific for the above-described  
35 altered superantigen toxins. For instance, an

- antibody can be raised against the complete toxin or against a portion thereof. Persons with ordinary skill in the art using standard methodology can raise monoclonal and polyclonal antibodies to the altered
- 5 superantigens of the present invention, or a unique portion of the altered superantigen. Materials and methods for producing antibodies are well known in the art (see for example Goding, in, Monoclonal Antibodies: Principles and Practice, Chapter 4, 1986).
- 10 The antibodies can be used in diagnostic assays for detection of superantigen toxin-associated infection. Neutralizing antibodies can be used in a therapeutic composition for the treatment of amelioration of anergy and/or for the treatment of a superantigen.
- 15 toxin-associated infection.

In a further embodiment, the present invention relates to a method for detecting the presence of superantigen-associated bacterial infections in a sample. Using standard methodology well known in the

20 art, a diagnostic assay can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), all or a unique portion of the altered superantigen described above, and

25 contacting it with the serum of a person suspected of having a superantigen-associated bacterial infection. The presence of a resulting complex formed between the altered superantigen toxin and antibodies specific therefor in the serum can be detected by any of the

30 known methods common in the art, such as fluorescent antibody spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of superantigen-associated bacterial infections.

In yet another embodiment, the present invention relates to a method for detecting the presence of superantigen toxin in a sample. Using standard methodology well known in the art, a diagnostic assay  
5 can be constructed by coating on a surface (i.e. a solid support) for example, a microtitration plate or a membrane (e.g. nitrocellulose membrane), antibodies specific for altered superantigen toxin, and contacting it with serum or tissue sample of a person  
10 suspected of having superantigen-associated bacterial infection. The presence of a resulting complex formed between toxin in the serum and antibodies specific therefor can be detected by any of the known methods common in the art, such as fluorescent antibody  
15 spectroscopy or colorimetry. This method of detection can be used, for example, for the diagnosis of superantigen-associated bacterial infection or disease such as food poisoning and toxic-shock syndrome or the detection of superantigen toxin in food and drink.

20 In another embodiment, the present invention relates to a diagnostic kit which contains altered superantigen toxin from a specific bacteria or several different superantigen toxins from bacteria and ancillary reagents that are well known in the art and  
25 that are suitable for use in detecting the presence of antibodies to superantigen toxin-associated bacteria in serum or a tissue sample. Tissue samples contemplated can be avian, fish, or mammal including monkey and human.

30 In yet another embodiment, the present invention relates to a vaccine for protection against superantigen toxin-associated bacterial infections. The vaccine can comprise one or a mixture of individual altered superantigen toxins, or a portion  
35 thereof. When a mixture of two or more different

altered superantigen toxin from different bacteria is used, the vaccine is referred to as a multivalent bacterial superantigen vaccine. The vaccine is designed to protect against the pathologies resulting from exposure to one or several related staphylococcal and streptococcal toxins. In addition, the protein or polypeptide can be fused or absorbed to other proteins or polypeptides which increase its antigenicity, thereby producing higher titers of neutralizing antibody when used as a vaccine. Examples of such proteins or polypeptides include any adjuvants or carriers safe for human use, such as aluminum hydroxide.

The staphylococcal enterotoxin (SE) serotypes SEA, SED, and SEE are closely related by amino acid sequence, while SEB, SEC1, SEC2, SEC3, and the streptococcal pyrogenic exotoxins B share key amino acid residues with the other toxins, but exhibit only weak sequence homology overall. However, there are considerable similarities in the known three-dimensional structures of SEA, SEB, SEC1, SEC3, and TSST-1. Because of this structural similarity, it is likely that polyclonal antibodies obtained from mice immunized with each SE or TSST-1 exhibit a low to high degree of cross-reaction. In the mouse, these antibody cross-reactions are sufficient to neutralize the toxicity of most other SE/TSST-1, depending upon the challenge dose. For example, immunization with a mixture of SEA, SEB, TSST-1 and SPEa was sufficient to provide antibody protection from a challenge with any of the component toxins, singly or in combination.

The likelihood of substantial antigen-cross-reactivity suggests that it may be possible to obtain immune protection for other (or perhaps all) staphylococcal superantigens by use of a minimal mixed

composition of vaccines. For the case of staphylococcal superantigens, a combination of the component vaccines from SEA, SEB, SEC-1 and TSST-1 should be sufficient to provide immune protection against SEA, SEB, SEC1-3, and TSST-1. The addition of SPEa component to the trivalent mixture will allow for sufficient protection against the streptococcal toxins SPEa and SPEc. Therefore, a multivalent vaccine consisting of the altered superantigen toxins from SEA, SEB, SEC-1, TSST-1, and SPEa as described above, is predicted to provide protective immunity against the majority of bacterial superantigen toxins.

The vaccine can be prepared by inducing expression of a recombinant expression vector comprising the gene for the altered toxin described above. The purified solution is prepared for administration to mammals by methods known in the art, which can include filtering to sterilize the solution, diluting the solution, adding an adjuvant and stabilizing the solution. The vaccine can be lyophilized to produce a vaccine against superantigen toxin-associated bacteria in a dried form for ease in transportation and storage. Further, the vaccine may be prepared in the form of a mixed vaccine which contains the altered superantigen toxin(s) described above and at least one other antigen as long as the added antigen does not interfere with the effectiveness of the vaccine and the side effects and adverse reactions, if any, are not increased additively or synergistically. Furthermore, the vaccine may be administered by a bacterial delivery system and displayed by a recombinant host cell such as *Salmonella* spp, *Shigella* spp, *Streptococcus* spp. Methods for introducing recombinant vectors into host cells and introducing host cells as a DNA delivery

system are known in the art [Harokopakis et al. (1997) *Infect. Immun.* **65**, 1445-1454; Anderson et al. (1996) *Vaccine* **14**, 1384-1390; Medaglini et al. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 6868-6872].

5       The vaccine may be stored in a sealed vial, ampule or the like. The present vaccine can generally be administered in the form of a liquid or suspension. In the case where the vaccine is in a dried form, the vaccine is dissolved or suspended in sterilized  
10 distilled water before administration. Generally, the vaccine may be administered orally, subcutaneously, intradermally or intramuscularly but preferably intranasally in a dose effective for the production of neutralizing antibody and protection from infection or  
15 disease.

      In another embodiment, the present invention relates to a method of reducing superantigen-associated bacterial infection symptoms in a patient by administering to said patient an effective amount  
20 of anti-altered superantigen toxin antibodies, as described above. When providing a patient with anti-superantigen toxin antibodies, or agents capable of inhibiting superantigen function to a recipient patient, the dosage of administered agent will vary  
25 depending upon such factors as the patient's age, weight, height, sex, general medical condition, previous medical history, etc. In general, it is desirable to provide the recipient with a dosage of the above compounds which is in the range of from  
30 about 1pg/kg to 10 mg/kg (body weight of patient), although a lower or higher dosage may be administered.

      In a further embodiment, the present invention relates to a therapeutic method for the treatment of diseases that may not be associated directly with  
35 superantigen toxins but which result in specific

nonresponsiveness of T-cell subsets or detection of abnormally low level of subsets in peripheral blood, said method comprising the administration of altered superantigen toxins, *in vivo* or *ex vivo*, such that T-cell subsets are expanded or stimulated. Diseases which cause anergy or nonresponsiveness of T-cells include, but are not limited to, infectious diseases and cancers. The desired clinical outcome such as an increase in detectable T cell subsets or in stimulation *ex vivo* of T-cells through their antigen receptors, such as by antigen or anti-CD3 antibody can be measured by standard clinical immunology laboratory assays.

In yet another embodiment, the present invention relates to a therapeutic method for the treatment of diseases associated with expanded or over-stimulated T-cell subsets, such as autoimmunity for example, said method comprising administration *in vivo* or *ex vivo*, of superantigen toxin altered in such a manner that only limited (1-3) T-cell subsets are stimulated but that MHC class II binding affinity still remains, such that anergy or inactivation of T-cells is produced. The desired clinical outcome can be measured as a reduction of circulating blood T-cells of the targeted subset(s) or diminished antigen or other antigen receptor-mediated-stimulatory responses by assays known in the art.

Described below are examples of the present invention which are provided only for illustrative purposes, and not to limit the scope of the present invention. In light of the present disclosure, numerous embodiments within the scope of the claims will be apparent to those of ordinary skill in the art.



The following Materials and Methods were used in the Examples that follow.

Structural comparisons

Primary protein structure data are available for  
5 several bacterial superantigens, including SEA, SED, SEB, SEC1-3, TSST-1. Superantigens for which structures were unavailable were modeled using comparative techniques (HOMOLOGY program; Biosym Technologies, Inc., San Diego, CA). Before x-ray  
10 crystallography data was available, SEA was modeled by using this method, and the model was in very close agreement with the experimentally determined structure. As an example, the amino acid sequence for SEA was aligned with the known structure of free and  
15 HLA-DR1 bound SEB, and the SEA molecule was built for both free and DR1-bound proteins. Loop segments of SEA were generated by a *de novo* method. Refinement of the modeled structures was carried out by means of molecular-dynamics simulations (DISCOVER, Biosym).  
20 The constructed free SEA molecule was immersed in a 5-Å layer of solvent water and the  $\alpha$ -carbon atoms lying in the structurally conserved regions were tethered to their initial positions during the simulations. For the bound SEA molecule, simulations were carried out  
25 by constructing an active-site region composed of part of the SEA molecule and the DR1 molecule inside a 10-Å interface boundary, as derived from the crystal structure of the DR1-SEB complex. Amino acid residues lying in the outer boundary were rigidly restrained at  
30 their initial positions. The active-site region was immersed in a 5-Å layer of water. Protein interactions were modeled by employing the consistent valence force field with a non-bonded cutoff distance of 11.0 Å. Simulations were initiated with 100 cycles  
35 of minimization using a steepest descent algorithm

followed by 100-ps relaxation (using a 1.0 fs timestep). Structural comparisons between SEB, SEC1, and TSST-1 were performed by using the crystal structures (Brookhaven data holdings) aligned according to common secondary structural elements and/or by sequence and structural homology modeling.

#### Site-specific mutagenesis

Site-specific mutagenesis was performed according to the method developed by Kunkel, using gene templates isolated from *Staphylococcus aureus* strains expressing SEA (FDA196E, a clinical isolate, Fraser, J.D. (1994) *Nature* **368**: 711-718), SEB (14458, clinical isolate), SEC1 (Toxin Technologies, Sarasota, FL), TSST-1 (pRN6550 cloned product, a clinical isolate, Kreiswirth, B. N. et al. (1987) *Mol. Gen. Genet.* **208**, 84-87), and SPEa (Toxin Technologies), respectively. Modified T7 polymerase (Sequenase, U.S. Biochemical Corp., Cleveland, OH) was used to synthesize second-strand DNA from synthetic oligonucleotides harboring the altered codon and single-stranded, uracil-enriched M13 templates. Mutagenized DNA was selected by transforming *E. coli* strain JM101. Alternatively, double stranded DNA was used as template for mutagenesis. Mutagenized sequences were confirmed by DNA sequencing (Sanger et al., 1977, *Proc. Natl. Acad. Sci. USA* **74**: 5463-5467; Sambrook et al., 1989) using synthetic primers derived from known sequences, or universal primers. The complete coding sequences were inserted into expression plasmids such as pUC19, pSE380 or pET21 for production in *E. coli* hosts.

### Protein purifications

The appropriate *E. coli* hosts were transformed with plasmids harboring the mutant toxin genes. In general, the bacteria were grown to an A600 0.5-0.6 in  
5 Terrific Broth (Difco Laboratories, Detroit, MI) containing 50 µg/mL ampicillin or kanamycin. Recombinant proteins were induced with isopropyl-β-D-thio-galactopyranoside (Life Technologies, Gaithersburg, MD) and recovered as cytoplasmic or  
10 bacterial periplasmic secretion products. Bacteria were collected by centrifugation, washed with 30 mM NaCl, 10 mM TRIS (pH 7.6), and pelleted by centrifugation and either lysed or osmotically shocked for collection of secreted proteins. Preparations  
15 were isolated by CM Sepharose ion-exchange chromatography, rabbit antibody (Toxin Technologies, Sarasota, FL) affinity columns, ion exchange HPLC or similar methods. In some cases partially purified superantigen was further purified by preparative  
20 isoelectric focusing (MinipHor; Rainin Instrument Company, Inc., Woburn, MA.). The MinipHor was loaded with the SEA-enriched fraction from CM Sepharose chromatography in a solution containing 10% (v/v) glycerol and 1% (v/v) pH 6-8 ampholytes (Protein  
25 Technologies, Inc., Tucson, AZ). The protein preparations were allowed to focus until equilibrium was reached (approximately 4 hr, 4°C). Twenty focused fractions were collected and aliquots of each were analyzed by SDS-polyacrylamide gel electrophoresis  
30 (SDS-PAGE) and immunoblotting. The SEA-containing fractions were pooled, and refocused for an additional 4 h. The fractions containing purified SEA were pooled and dialyzed first against 1 M NaCl (48 h, 4°C) to remove ampholytes, and then against PBS (12 h,  
35 4°C). Legitimate amino-terminal residues were

confirmed by protein sequencing. Precise measurements of protein concentrations were performed by immunoassay using rabbit antibody affinity-purified with the wild-type superantigens and by the  
5 bicinchoninic acid method (Pierce, Rockford, IL) using wild-type protein as standards. All protein preparations were >99% pure, as judged by SDS-PAGE and Western immunoblots. In some cases, as when used for lymphocyte assays, bacterial pyrogens were removed by  
10 passing the protein preparations over Polymyxin B affinity columns.

#### Binding of superantigens to HLA-DR1

The DR1 homozygous, human B-lymphoblastoid cell  
15 line LG2 or L cells transfected with plasmids encoding HLA-DR1 $\alpha\beta$  were used in the binding experiments. Cells were incubated 40 min (37°C) with wild-type or mutant superantigen in Hanks balanced salt solution (HBSS) containing 0.5% bovine serum albumin. The cells were  
20 washed with HBSS and then incubated with 5  $\mu$ g of specific rabbit antibody (Toxin Technology, Sarasota, FL) for 1 h on ice. Unbound antibody was removed, and the cells were incubated with FITC-labelled goat anti-rabbit IgG (Organon Teknika Corp., Durham, N.C.) on  
25 ice for 30 min. The cells were washed and analyzed by flow cytometry (FACScan; Becton Dickinson & Co., Mountain View, CA). Controls consisted of cells incubated with affinity purified anti-toxin and the FITC labelled antibody without prior addition of  
30 superantigen.

#### Lymphocyte proliferation

Human peripheral blood mononuclear cells were purified by Ficoll-hypaque (Sigma, St. Louis, MO)

buoyant density gradient centrifugation. Genes encoding the human MHC class II molecules DR1 $\alpha\beta$  (DRA and DRB1\*0101 cDNA [Bavari and Ulrich (1995) *Infect. Immun.* 63, 423-429] were cloned into the eukaryotic expression vector pRC/RSV (Invitrogen, Carlsbad, CA), and mouse L cells were stably transfected. The transfectants were selected by fluorescence-activated cell sorting (EPICS C; Coulter Corp., Hialeah, FL) using rabbit anti-DR $\alpha\beta$  antisera and FITC-goat anti-rabbit IgG, to produce cells that expressed a high level of DR $\alpha\beta$ .  $1 \times 10^5$  cells/well of a 96-well plate were irradiated (15,000 Rad), and wild-type or mutant SE, was added. After a brief incubation period (45 min, 37°C), unbound SE was rinsed from the culture plates using warm media. The cells were cultured in RPMI-1640 (USAMRIID) with 5% FBS for 72 h, and pulsed-labelled for 12 h with 1 $\mu$ Ci [ $^3$ H]-thymidine (Amersham, Arlington Heights, IL). Cells were harvested onto glass fiber filters, and [ $^3$ H]-thymidine incorporation into the cellular DNA was measured by a liquid scintillation counter (BetaPlate, Wallac Inc., Gaithersburg, MD). Splenic mononuclear cells or human peripheral blood mononuclear cells were obtained by buoyant density centrifugation (Histopaque; Sigma Chemical Comp.) and washed three times. The cells were resuspended in medium containing 5% fetal bovine serum (FBS), and 100  $\mu$ l ( $4 \times 10^5$  cells) of the cell suspension was added to triplicate wells of 96-well flat bottom plates. The mononuclear cells were cultured (37°C, 5% CO<sub>2</sub>) with WT or mutant SEA. After 3 days the cultures were pulsed (12h) with 1  $\mu$ Ci/well of [ $^3$ H]thymidine (Amersham, Arlington Heights, IL) and incorporated radioactivity was measured by liquid scintillation.

Gel electrophoresis and immunoblotting analysis.

The protein preparations were analyzed by SDS-PAGE (12%) and stained with Coomassie Brilliant Blue R-250 (Sigma Chemical Comp. St Louis, MO) in methanol (10% v/v) acetic acid (10% v/v). The proteins separated by SDS-PAGE (not stained) were transferred to nitrocellulose membranes (Bio-Rad Lab. Inc., Melville, NY) by electroblotting, and the membranes were then blocked (12 h, 4°C) with 0.2% casein in a buffer consisting of 50 mM sodium phosphate, 140 mM sodium chloride, pH 7.4 (PBS). The membrane was then incubated (1 h, 37°C, shaking) with 2 µg/mL of affinity-purified anti-toxin antibody (Toxin Technology, Sarasota, FL) in PBS with 0.02% casein. After the membranes were thoroughly washed, peroxidase-conjugated goat anti-rabbit IgG (Cappel/Organon Teknika Corp., West Chester, PA) was added (1:5,000) and the membranes were incubated for 1 h (37°C) with shaking. The unbound antibody was removed by washing with PBS and bound antibody was visualized by using a Bio-Rad peroxidase development kit (Biorad, Hercules, CA). For quantitation, dilutions of wild-type preparations were immobilized on nitrocellulose membranes by using a Slot-Blot apparatus (Bio-Rad). The membrane was removed from the Slot-Blot apparatus and unreacted sites were blocked (12h, 4°C) with 0.2% casein in PBS. After washing once with the PBS, the membrane was incubated (1h, 37°C) with 2 µg/mL rabbit affinity purified anti-toxin antibody (Toxin Technology) in PBS that contained 0.02% casein. After four washes, the bound rabbit antibody was reacted with goat anti-rabbit IgG conjugated with horseradish peroxidase (1 h, 37°C) and the blots were developed using enhanced

chemiluminescence (ECL; Amersham Life Sciences, Arlington Heights, IL) or similar methods. The amount of mutant protein was measured by densitometry (NIH Image 1.57 software, National Institutes of Health, Bethesda, MD) of exposed X-ray film. Standard curves were prepared by plotting the mean of duplicate densitometric readings for each dilution of toxin standard. The resulting values were fitted to a straight line by linear regression. Concentrations of proteins were determined by comparing mean values of various dilutions of the mutant to the standard curve.

#### Biological activities and Immunizations.

Male C57BL/6 mice, 10 to 12-weeks old, were obtained from Harlan Sprague-Dawley, Inc. (Frederick Cancer Research and Development Center, Frederick, MD). The lethal effect of WT or mutant SEA was evaluated as described in Stiles et al. (1993) *Infect. Immun.* **61**, 5333-5338. For immunizations, mice were given by interperitoneal (ip) injections either 2 or 10 µg of WT or mutant toxin in 100 µl of adjuvant (RIBI, Immunochem Research, Inc. Hamilton, MT or alum), or adjuvant only, and boosted (ip) at 2 and 4 weeks. Serum was collected from tail veins one week after the last immunization. Mice were challenged 2 weeks after the last injection with toxin and lipopolysaccharide (LPS, 150 µg) from *E. coli* 055:B5 serotype (Difco Laboratories, Detroit, MI). Challenge controls were adjuvant-immunized or non-immunized mice injected with both agents (100% lethality) or with either wild type toxin or LPS. No lethality was produced by these negative controls. Monkeys were immunized with the antigen in the right leg, caudal thigh muscles. Each received three intramuscular immunizations with a superantigen vaccine plus

adjuvant. Control monkeys received 0.5 ml total volume of adjuvant (Alhydrogel, Michigan Department of Public Health) and sterile PBS using the same techniques and equipment as the immunized monkeys.

5 Immunizations were administered  $28 \pm 2$  days apart and consisted of 20  $\mu$ g of the vaccine in adjuvant in a total volume of 0.5 ml. Immunizations were administered on day 0,  $28 \pm 2$ , and  $56 \pm 2$  using a 23-27 ga 1/2-5/8" needle attached to a 1 ml tuberculin syringe

10 into the caudal thigh.

#### Antibody assay.

Microtiter plates were coated with 1  $\mu$ g/well of WT toxin in 100  $\mu$ l of PBS (37°C, 2 h). After antigen

15 coating, the wells were blocked with 250  $\mu$ l of casein 0.2% in PBS for 4 h at 37°C and then washed four times with PBS containing 0.2% Tween 20. Immune or nonimmune sera were diluted in PBS containing 0.02% casein and 100  $\mu$ l of each dilution was added to

20 duplicate wells. After each well was washed four times, bound antibody was detected with horse radish peroxidase (Sigma Chemical Comp., St. Louis, MO) labelled goat anti-species specific IgG (37°C, 1 h), using O-phenylenediamine as the chromogen. Mean of

25 duplicates OD (absorbance at 490 nm) of each treatment group was obtained and these data were compared on the basis of the inverse of the highest serum dilution that produced an OD reading four times above the negative control wells. For negative controls,

30 antigen or serum was omitted from the wells.

#### Superantigen binding and TCR subset analysis.

Cells from the mouse B-lymphoma line A20 (ATCC, Rockville, MD) ( $2-4 \times 10^5$  cells) were incubated (40 min



at 37°C) with WT or mutant toxin in Hanks balanced salt solution containing 0.5% bovine serum albumin (HBSS, USAMRIID). The cells were washed with HBSS and incubated with 5 µg of affinity-purified anti-toxin  
5 antibody in HBSS (4°C, 45 min). Unbound antibody was removed and the bound antibody was detected with fluorescein isothiocyanate (FITC)-labelled, goat anti-rabbit IgG (Organon Teknika Corp., Durham, NC). Unbound antibody was removed and the cells were  
10 analyzed by with a FACSORT flow cytometer (Becton Dickinson & Co. , Mountain View, CA).

For TCR subset analysis, splenic mononuclear cells were obtained from mice immunized with WT or mutant toxin. The mononuclear cells were incubated  
15 (37°C) with WT toxin (100 ng/mL) for 5 days and then cultured in 85% RPMI-1640, 10% interleukin-2 supplement (Advanced Biotechnologies Inc., Columbia, MD) with 5% FBS for an additional 5 days. The T cells were washed twice and stained with anti-TCR  
20 (Biosource, Camarillo, CA) or anti-Vβ specific TCR (Biosource, Camarillo, CA) (45 min, 4°C). All cells analyzed were positive for T cell marker CD3+ and expressed the CD25 activation marker (data not shown). Controls were incubated with an isotype matched  
25 antibody of irrelevant specificity. Unreacted antibody was removed, and the cells were incubated with an FITC-labelled, anti-mouse IgG (Organon Teknika Corp, Durham, NC) on ice for 30 min. The cells were washed and analyzed by flow cytometry (FACSORT).

30

LPS potentiation of SE toxicity in mice.

C57BL/6 or BALB/c mice weighing 18-20 g (Harlan Sprague Dawley, Inc., Frederick Cancer Research and Development Center, Frederick, MD) were each injected  
35 intraperitoneally (i.p.) with 200 µl of PBS containing

varying amounts of SEA, SEB, or SEC1, TSST-1, or SPEa followed 4 h later with 75 or 150  $\mu$ g of LPS (200  $\mu$ l/i.p.). Controls were each injected with either SE (30 mg) or LPS (150 mg). Animals were observed for 72 h after the LPS injection. Calculations of LD50 were done by Probit analysis using 95% fiducial limits (SAS Institute Inc., Cary, NC).

The biological effects of SEA and SEB were also tested in transgenic C57BL/6 mice (GenPharm International, Mountain View, CA) deficient in MHC class I or II expression [Stiles et al. (1993) *Infect. Immun.* **61**, 5333-5338], as described above, using a single dose of toxin (30  $\mu$ g/mouse). Genetic homozygosity was confirmed by Southern analysis of parental tail DNA, using  $\beta$ 2 microglobulin and MHC class II  $\beta$  DNA probes.

#### Detection of cytokines in serum.

Mice (n=18 per group) were injected with toxin (10  $\mu$ g), LPS (150  $\mu$ g), or toxin plus LPS. Sera were collected and pooled from three mice per group at each time point (2, 4, 6, 8, 10, 22 h) after LPS injection. Sera were collected at various time points following toxin injection (-4 h, or 4h before LPS injection, for data tabulation). Collection of LPS control sera began at the time of injection (0 h).

Serum levels of TNF $\alpha$  and IL- $\alpha$  were detected by an enzyme linked immunosorbent assay (ELISA). TNF $\alpha$  was first captured by a monoclonal antibody against mouse TNF $\alpha$  (GIBCO-BRL, Grand Island, NY) and then incubated with rabbit anti-mouse TNF $\alpha$  antibody (Genzyme, Boston, MA). The ELISA plate was washed and peroxidase conjugate of anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) added to the wells. After washing the plate and adding substrate (Kirkegaard and

- Perry, Gaithersburg, MD), TNF $\alpha$  concentrations were measured using the mean A450 reading of duplicate samples and a standard curve generated from recombinant mouse TNF $\alpha$  (GIBCO-BRL). Serum levels of
- 5 IL-1 $\alpha$  were determined from the mean reading of duplicate samples with an ELISA kit that specifically detects murine IL-1 $\alpha$  (Genzyme, Boston, MA). The standard error of the mean (SEM) for TNF $\alpha$  and IL-1 $\alpha$  readings was +/- 5%.
- 10 Quantitation of IL-6 and IFN $\gamma$  were measured by bioassays [See et al. (1990) *Infect. Immun.* **58**: 2392-2396]. An IL-6 dependent cell line, 7TD1 (kindly provided by T. Krakauer), was used in a proliferative assay with serial two-fold dilutions of serum samples
- 15 assayed in triplicate. Proliferation of 7TD1 cells in a microtiter plate was measured by uptake of [ $^3$ H]-thymidine (1  $\mu$ Ci/well; Amersham, Arlington Heights, IL) and the activity of IL-6 from serum was compared to a recombinant mouse IL-6 standard (R and D Systems,
- 20 Minneapolis, MN) as previously described [See et al. (1990) *Infect. Immun.* **58**: 2392-2396]. The SEM of triplicate samples was +/- 10%.
- IFN $\gamma$  was measured by the reduction of vesicular stomatitis virus (New Jersey strain) cytopathic
- 25 effects on L929 cells, as previously described [Torre et al. (1993) *J. Infect. Dis.* **167**, 762-765]. Briefly, serial two-fold dilutions of serum were made in duplicate and added to microtiter wells containing L929 cells ( $5 \times 10^4$ /well). After incubating 24 h,
- 30 virus ( $5 \times 10^5$  PFU/well) was added and the cytopathic effects measured at 48 h by absorbance readings (570 nm) of reduced 3-[4, 5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (Sigma). The activity of each serum sample was determined using recombinant

mouse IFN $\gamma$  as a standard (Biosource, Camarillo, CA).  
The SEM of duplicate samples was  $\pm$  5%.

#### EXAMPLE 1

5        Molecular modelling and structural studies of  
         staphylococcal and streptococcal superantigens:  
         bacterial superantigens share common 3-dimensional  
         structure.

         Comparison of amino acid sequences (Fig. 1)  
10       suggested that bacterial superantigens fall into  
         groups consisting of (1) SEA, SED and SEE, (2) SEB,  
         staphylococcal enterotoxins C1-C3 (SEC1-3), the  
         streptococcal pyrogenic exotoxins A (SPE-A) and C  
         (SPE-C), (3) TSST-1 and (4) the exfoliative toxins  
15       (ETA, ETB) and streptococcal pyrogenic exotoxin B  
         (SPE-B), which are the most distant from the others in  
         sequence. Although not available to the inventor when  
         the inventions were first conceived and proof of  
         principle was obtained, the x-ray crystallographic  
20       structures of several bacterial superantigens are now  
         known. Diverse superantigens, such as SEB and TSST-1,  
         appear to have little sequence in common, yet they  
         exhibit homologous protein folds composed largely of  $\beta$   
         strands [Prasad, G.S. et al. (1993) *Biochemistry* 32,  
25       13761-13766; Acharya, R.K. et al. (1994) *Nature* 367,  
         94-97; Swaminathan, S. et al. (1992) *Nature* 359, 801-  
         806] within two distinct domains. Differences between  
         the proteins are located primarily in highly variable  
         regions comprised of several surface loops, such as  
30       the disulfide-bonded loop which is absent from TSST-1  
         and at the amino terminus.

         The X-ray crystal structures of SEB and TSST-1  
         complexed with HLA DR1 are known [Kim, J. et al.  
         (1994) *Science* 266, 1870-1874 ; Jardetzky, T.S. et al.

(1994) *Nature* 368, 711-718] and this data was useful to fully explain our results concerning attenuation of the superantigens by site-specific mutagenesis. The region of HLA DR1 that contacts SEB consists  
5 exclusively of  $\alpha$  subunit surfaces. The main regions of SEB involved are two conserved sites: a polar pocket derived from three  $\beta$  strands of the  $\beta$  barrel domain and a highly solvent-exposed hydrophobic reverse turn. The polar binding pocket of SEB  
10 contains a glutamate and two tyrosines that accommodate Lys39 of the  $\alpha$  subunit of HLA DR1, while the hydrophobic region consists of a leucine and flanking residues that make several contacts with the HLA DR $\alpha$  chain. The HLA DR1 binding sites of both  
15 TSST-1 and SEB overlap significantly. The hydrophobic binding contacts of other SAg with the HLA DR $\alpha$  chain have been proposed [Ulrich et al. (1995) *Nature, Struct. Biol.* 2, 554-560] to be similar to those found in SEB and TSST-1. A motif consisting of a leucine in  
20 a reverse turn [Ulrich et al. (1995), supra] is conserved among bacterial superantigens and may provide the key determinant (hydrophobic or otherwise) for binding HLA-DR. However, TSST-1 does not have a highly charged residue in the polar pocket that  
25 interacts with Lys39 of the HLA DR $\alpha$  chain and uses an alternative conformational binding mode that allows TSST-1 to interact with HLA DR1  $\beta$ -chain residues and the carboxy-terminal region of the antigenic peptide.

Both SEA and SEE bind to the  $\beta$  subunit of DR by  
30 means of a single zinc atom [Fraser, J.D. et al. (1992) *Proc. Natl. Acad. Sci. USA* 89, 5507-5511]. The amino-terminal domain of SEA interfaces with the HLA DR $\alpha$  chain [Ulrich et al. (1995), supra], while SEA C-terminal domain residues His187, His225 and Asp227  
35 form a zinc-coordination complex, likely with His-81

from the  $\beta$  chain of an adjoining HLA DR molecule. However, our results have shown that binding of superantigen to the HLA DR $\beta$  subunit does not directly stimulate T cells [Ulrich et al. (1995), supra] but  
5 increases the potential of the bound SEA to interact with the  $\alpha$  chain of another HLA DR, thus increasing the biological potency.

#### EXAMPLE 2

10       Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: A detailed protein structure analysis of SEB and SEA suggested that all bacterial superantigens have a common mechanism for binding MHC class II receptors.

15       A least-squares superimposition of the unbound molecules of modeled SEA and the crystal structure of SEB, aligned according to their structurally conserved  $\alpha$ -helical and  $\beta$ -strand regions, exhibited a global folding pattern which is very similar. Differences  
20 between the two structures are calculated to be located primarily in loops of low sequence homologies, with the largest positional deviations occurring between structurally conserved regions of residues 18-20, 30-32, 173-181, 191-194, and the cysteine-loop  
25 region (90-111). Only one of these regions in SEB makes significant contact (residue Y94 in particular) with the HLA-DR1 molecule [Jardetzky, T.S. et al. (1994) *Nature* **368**, 711-718].

30       The binding interface between SEB and HLA-DR1 consists principally of two structurally conserved surfaces located in the N-terminal domain: a polar binding pocket derived from three  $\beta$ -strand elements of the  $\beta$ -barrel domain and a hydrophobic reverse turn. The binding pocket of SEB contains residues E67, Y89

and Y115, and binds K39 of the DR $\alpha$  subunit. For SEA, the binding interface with the DR molecule is modeled to contain a similar binding pocket consisting of residues D70, Y92 and Y108. Mutation of residue Y89 in SEB or Y92 in SEA to alanine (Fig. 2) resulted in 100-fold reduction in DR1 binding. The substitution of alanine for Y89 in SEB and Y92 in SEA eliminates the hydrogen bond with K39 and disrupts packing interactions with adjacent protein residues. Modeling of the SEA mutant Y92A predicts an increase in solvent-accessible surface area for Y108 by a factor of two greater than the wild-type structure, allowing the formation of a hydrogen bond to the carboxylate group of D70 and thus disrupting key anchoring and recognition points for HLA-DR1. This effect is expected to be somewhat less in SEB due to the longer side chain at E67. Substitution of SEB Y115 with alanine also resulted in 100-fold reduction of binding. In contrast, the same replacement of Y108 in SEA yielded little to no change in DR1 binding (Fig. 2a), suggesting the primary importance of SEA residues Y92 and D70 for stabilizing interactions with K39. The K39 side chain of DR $\alpha$  forms a strong ion-pair interaction with the SEB E67 carboxylate group and hydrogen bonds with the hydroxyl groups of Y89 and Y115. Substitution of SEB E67 by glutamine reduced binding affinity by 100-fold (Fig. 2), reflecting the replacement of the strong ionic bond with a weaker hydrogen bond. To optimize ion-pair interactions of the analogous SEA site, the shorter carboxylate side chain of D70 is predicted to shift K39 of DR $\alpha$ , weakening interactions with SEA Y108. The substitution of alanine for SEA Y108 is thus more easily accommodated than the homologous substitution of SEB Y115, without loss in DR1 binding.

Comparisons of the polar pocket with other bacterial superantigens were then made. SEC1-3 and SPE-A have conserved the critical DR1 binding-interface residues (**Fig. 1**), and share with SEB and SEA secondary structural elements of the DR1-binding surfaces. Asparagine in SED (N70) replaces the acidic side chain present in SEA, SEB, SPE-A and SEC1-3. Accordingly, for SED the salt bridge of the polar pocket is likely to be replaced by a hydrogen bond.

Overall DR1 affinities for SED and SEA appeared to be equivalent (**Fig. 2b**), indicating that other interactions may compensate for the absence in SED of the ion-pair found in the other superantigens. For the case of TSST-1, mutating DR $\alpha$  residues K39 to serine or M36 to isoleucine has been shown to greatly reduce binding [Panina-Bordignon et al. (1992) *J. Exp. Med.* **176**: 1779-1784]. Although primarily hydrophobic, the critical TSST-1 structural elements are conserved with the SEA and SEB polar binding pocket. SEB residues Y89 and Y115 are homologous to T69 and I85 in TSST-1, respectively, and SEB E67 is replaced by I46. These TSST-1 residues are positioned in a conserved  $\beta$ -barrel domain found in both SEB and SEA. However, the TSST-1 site lacks polarity equivalent to SEB/SEA, and hydrogen bonding with the hydroxyl of TSST-1 residue T69 would require that DR $\alpha$  K39 extend 5 Å into the pocket. TSST-1 binding utilizes an alternative strategy [Kim et al. (1994) *Science* **266**: 1870-1874] consisting of hydrophobic contacts centered around residue I46, and potential ionic or hydrogen bonds bridging DR $\alpha$  residues E71 and K67 to R34 and D27, respectively, of TSST-1.

The hydrophobic region of the binding interface between SEB and the HLA-DR1 molecule consists of SEB residues 44-47, located in a large reverse turn



connecting  $\beta$ -strands 1 and 2 of SEB. These residues appear to make strong electrostatic interactions with DR $\alpha$  through their backbone atoms. The mutation of L45 to an arginine reduced overall HLA-DR1 binding greater than 100-fold (**Fig. 2b**), attributable to the less energetically favorable insertion of a highly charged residue into a hydrophobic depression on the DR1 molecule. The modeled DR1-SEA complex presents similar interactions with the SEA backbone atoms, with the exception of a glutamine (Q49) replacing SEB Y46. Mutation of L48 to glycine in SEA (homologous to L45 of SEB) has been reported to decrease T-cell responses. SEB L45 and the comparable L30 of TSST-1 are the most extensively buried residues in the DR1 interface. The leucine is conserved among the bacterial superantigens (**Fig. 3**) and may provide the necessary hydrophobic structural element for surface complementarity with DR1, consistent with the mutagenesis data for SEB and SEA.

The inventor has performed similar structure and function studies with TSST-1, SEC1 and SPE-A.

### EXAMPLE 3

Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: Some interactions of bacterial superantigens with MHC class II receptors are not conserved but are less important than the hydrophobic loop and polar pocket binding sites.

In determining the overall affinity of the superantigen for DR1, a contributory role is played by structural variations around the common binding motifs. A short, variable structured, disulfide-bonded loop is found in SEA and a homologous longer

- loop in SEB. The SEB residue Y94, contained within this loop, forms hydrophobic interactions with L60 and A61 of the DR $\alpha$  subunit. Replacement of Y94 with alanine partially inhibits DR1 binding (Fig. 2a,b).
- 5 An alanine is found in SEA (A97) and SEE at the position equivalent to SEB Y94, and mutating this residue in SEA to tyrosine results in disrupted instead of stabilized interactions with DR1 (Fig. 2a). Although the disulfide loops differ in structure
- 10 between SEA and SEB, A97 apparently contributes to the DR $\alpha$  binding interface in a manner similar to Y94 of SEB. Because TSST-1 lacks a disulfide loop, similar contacts with DR $\alpha$  are replaced by interactions with  $\beta$ -strands of TSST-1. In a like manner, the absence of a
- 15 salt bridge between the residues K39 of DR $\alpha$  and E67 of SED is apparently compensated for by stabilizing interactions occurring outside of the otherwise conserved dominant binding surfaces (Fig. 2a).

20

#### EXAMPLE 4

Molecular modelling and structural studies of staphylococcal and streptococcal superantigens: Superantigen interactions with T-cell antigen receptors.

- 25 The amino acid residues in contact with TCR are located in regions of high sequence variability, presenting a unique surface for interaction with the TCR. Residues implicated in TCR interactions by mutagenesis of SEA and SEB reside in variable loop
- 30 regions, while TSST-1 mutants that affect TCR binding are mainly located in an  $\alpha$  helix [Acharya, R.K. et al. (1994) *Nature* **367**, 94-97; Kim, J. et al. (1994) *Science* **266**, 1870-1874]. Specifically, mutations that diminish T-cell receptor recognition of SEB include

residues N23, Y61, and the homologous SEA N25 or Y64 (Fig. 2c). SEA residues S206 and N207 also control T-cell responses [Hudson, et al. (1992) *J. Exp. Med.* 177: 175-184]. Mutants of the polar binding pocket, SEA Y92A and SEB Y89A, equivalently reduced T-cell responses (Fig. 2c), reflecting the observed decreases in DR1-binding (Fig. 2a, b). While supporting reduced T-cell responses, mutants SEA Y64A and SEB Y61A retained normal affinities for DR1 (Fig. 2a-c).

#### EXAMPLE 5

##### Animal models for determining biological activity of bacterial superantigens: Mouse.

When compared to primates, mice are not very susceptible to the toxic effects of SE, and we therefore sought to increase sensitivity with a potentiating dose of lipopolysaccharide (LPS) from Gram-negative bacteria [Stiles et al. (1993) *Infect. Immun.* 61, 5333-5338]. There was no apparent effect in control animals injected with any of the SE (up to 30 µg/mouse) or LPS (150 µg/mouse) alone (Table 1). Incremental injections of LPS were also not lethal, when given in amounts up to 250 µg/mouse (data not shown). However, mice died between 24-48 h after SE and LPS were given to the same animal (Table 1). SEA was much more toxic than either SEB or SEC1 and the calculated LD50 (µg toxin/kg) of SEA, SEB, and SEC1 with 95% fiducial limits was 18.5 (6.5, 38.5), 789.0 (582.5, 1044.5), and 369.0 (197.5, 676.0), respectively.

TABLE 1. Titration of SEA, SEB, and SEC<sub>1</sub> in the C57BL/6 mouse lethality assay

Stimulus <sup>a</sup>	% Lethality (no. of mice tested) with the following dose of SE, in micrograms/mouse <sup>b</sup> :			
	30	10	1	0.1
SEA + LPS	93 (15) <sup>b</sup>	85 (20)	80 (15)	20 (10)
SEB + LPS	80 (15)	27 (15)	0 (15)	0 (15)
SEC <sub>1</sub> + LPS	80 (10)	60 (10)	10 (10)	0 (10)

<sup>a</sup>LPS was injected into each mouse (150ug) 4 h after the SE injection. Control mice injected with 150 ug of LPS (n=20) or 30 ug of SEA, SEB, or SEC<sub>1</sub> (n=10) survived.

<sup>b</sup>Results are from a combination of separate experiments with five mice per experiment.

The role of MHC class I and class II molecules in SE toxicity, potentiated by LPS, was addressed by using transgenic, MHC-deficient mice (Table 2). Class II-deficient animals were unaffected by a dose of SE (30 µg) plus LPS (150 µg) that was lethal for 93% of wild-type and 30% of class I-deficient mice. Mononuclear cells from class II-deficient animals were not able to present SEA, as measured by proliferative responses. MHC class I-deficient cells were functional in supporting T-cell proliferation, but at levels <30% of the proliferative response supported by MHC-wild-type presenting cells (Table 3). Cell surface expression levels were normal, when compared to nontransgenic C57BL/6, for A<sup>b</sup> in class I-deficient mice, and K<sup>b</sup>/D<sup>b</sup> in class II-deficient mice. The T-cell responses of MHC class I- or class II-deficient mice were essentially equivalent to wild-type when SEA

was presented by mononuclear cells expressing both class I and II molecules (Table 3).

TABLE 2. Lethality of SEA and SEB in C57BL/6 mice

5 lacking MHC class I or class II

Stimulus <sup>a</sup>	% Lethality (no. of mice tested) with the following MHC class phenotype		
	I*II <sup>+</sup>	I <sup>+</sup> II <sup>+</sup>	I <sup>+</sup> II <sup>+</sup>
10 SEA + LPS	30(10)	0(5)	93(15)
SEA + LPS	ND <sup>b</sup>	0(5)	80(15)
SEA only	0(2)	0(2)	0(2)
SEB only	ND <sup>b</sup>	0(2)	0(2)
15 LPS only	0(5)	0(5)	0(5)

<sup>a</sup> Mice were injected with 30 ug of SEA or SEB and, 4h later, with 150 ug of LPS, as indicated. Control mice were injected with only SEA, SEB, or LPS.

20 <sup>b</sup> ND, not determined.

Table 3. Mouse T-cell responses to SEA are MHC class II-dependent

T-cell/APC source <sup>3</sup>	T-cell responses <sup>1</sup>	
	0.1 ug/ml SEA	1 ug/ml SEA
30 Wild-type C57/BL6 mouse/autologous	430,000 cpm <sup>2</sup>	700,000 cpm
MHC class I knock-out	117,000 cpm	167,000 cpm
35 C57/BL6 mouse/autologous		
MHC class II knock-out	8,000 cpm	33,000 cpm
40 C57/BL6 mouse/autologous		
Wild-type C57/BL6 mouse/wild-type	305,000 cpm	307,000 cpm
MHC class I knock-out	420,000 cpm	445,000 cpm
45		

C57/BL6 mouse/wild-type

MHC class II

5	knock-out C57/BL6 mouse/wild-type	310,000 cpm	322,000 cpm
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<sup>1</sup>Cultures of mononuclear cells derived from mouse spleens, cultured for 3 d with the indicated amount of SEA.

10 <sup>2</sup>Data represent the mean of triplicate determinations (<10 SEM) of [<sup>3</sup>H]thymidine incorporation.

15 <sup>3</sup>Antigen presenting cells (APC) were isolated from spleens of the indicated mouse strain and added to cultures.

The serum levels of TNF $\alpha$ , IL-1 $\alpha$ , IL-6, and IFN $\gamma$  in mice injected with SEA, LPS, or SEA plus LPS were measured at various times following injection (Fig. 4). Compared to mice injected with either SEA or LPS alone, the serum levels of TNF $\alpha$ , IL-6, and IFN $\gamma$  had increased 5-, 10-, and 15-fold, respectively, in animals given SEA plus LPS. SEA alone did not elicit any detectable increase of TNF $\alpha$ , IL-6, or IFN $\gamma$  above background. In contrast to the other cytokines, IL-1 $\alpha$  levels in mice injected with SEA plus LPS resulted in a simple additive effect.

Serum levels of TNF $\alpha$ , IL-6, and IFN $\gamma$  were maximal 2-4 h after the LPS injection, but returned to normal by 10 h. The concentration of IL-1 $\alpha$  in mice given SEA plus LPS had also peaked 2 h after the LPS injection, but stayed above background for the remaining determinations. Levels of IL-1 $\alpha$  in mice given only LPS or SEA peaked at 4 and 6 h, respectively. Unlike profiles for other cytokines, the highest amount of IL-1 $\alpha$  in mice injected with SEA and LPS corresponded to the peak stimulated by SEA, but not LPS.

This animal model was used in various stages of developing the inventions, as a means of assessing the physiological activity of mutated superantigens.

Control animals survived the maximum dose of either SE or LPS, while mice receiving both agents died. Wild-type SEA was 43-fold more potent than SEB and 20-fold more potent than SEC1. By using BALB/c mice the toxicity of SEB was 10-20 fold higher. These data confirmed that the toxicity of SE was mainly exerted through a mechanism dependent on expression of MHC class II molecules and was linked to stimulated cytokine release. Thus this was a relevant preclinical model that could be used to predict human responses.

#### EXAMPLE 6

##### Animal models for determining biological activity of bacterial superantigens: Rhesus monkey

The physiological responses of the rhesus monkey to bacterial superantigens is probably identical to humans, with the exception of sensitivity [Bavari and Ulrich (1995) *Clin. Immunol. Immunopath.* 76:248]. Generally SEB intoxicated monkeys developed gastrointestinal signs within 24 hours post-exposure. Clinical signs were mastication, anorexia, emesis and diarrhea. Following mild, brief, self-limiting gastrointestinal signs, monkeys had a variable period of up to 40 hours of clinical improvement. At approximately 48 hours post-exposure, intoxicated monkeys generally had an abrupt onset of rapidly progressive lethargy, dyspnea, and facial pallor. If given a lethal dose, death occurs within four hours of onset of symptoms. Only SEB has been used in challenges of rhesus monkeys to determine physiological/pathological effects. Human responses to bacterial superantigens are characterized by a rapid drop in blood pressure, elevated temperature,

and multiple organ failure-the classical toxic shock syndrome (TSS). However, the respiratory route of exposure may involve some unique mechanisms. The profound hypotension characteristic of TSS is not  
5 observed, and respiratory involvement is rapid, unlike TSS. Fever, prominent after aerosol exposure, is generally not observed in cases of SEB ingestion.

#### EXAMPLE 7

10     Targeting receptor interactions to develop vaccines.

      The SEA mutants Y92A, with reduced DR1 binding, and Y64A, with reduced TCR interactions, and K14E with wild-type (control) activity were used to determine  
15 the correct receptor to target for vaccine development. The binding of WT or mutant SEA was evaluated with the MHC class II expressing murine B-cell lymphoma cell line A20 (Table 4). The binding  
20 affinity of WT SEA to mouse MHC class II (H-2<sup>d</sup>) molecules was lower than that observed with human MHC class II expressing cells, reflecting the reduced toxicity that bacterial SAgS exert in mice. WT SEA, Y64A and K14E all had the same relative affinity to  
25 mouse MHC class II molecules. Similar to the results obtained with human MHC class II molecules, the Y92A mutant exhibited substantially reduced binding to A20 cells (Table 4).

30

35



Table 4. Biological activity of superantigen vaccines

	toxin	T-cell anergy <sup>1</sup>	MHC classII binding <sup>2</sup>	T-cell response
5	SEA			
	wild type	++++	+++	+++
10	TCR attenuated Y64A	+	+++	+/-
	MHC attenuated Y92A	-	+/-	+/-
15	Control K14E	++++	+++	+++

<sup>1</sup>Based on attenuation of T-cell response to wild-type SEA in mice immunized with the mutant or wild-type SEA.

<sup>2</sup>Binding to the mouse MHC class II+ A20 cells, measured by flow cytometry

- 25 The effect of WT SEA or site-specific SEA mutants on splenic mononuclear cells obtained from nonimmunized C57BL/6 (H-2<sup>b</sup>) mice is summarized in Table 4. Both WT SEA and the control mutant K14E were potent T cell activators, effective at minimal
- 30 concentrations of 10 to 100 pg/mL. However, T-cell responses to Y92A were reduced at least 100-fold, compared to SEA wild type, while Y64A-stimulated responses were slightly higher than Y92A. These results confirmed that attenuation of superantigen
- 35 binding to either MHC class II or TCR molecules resulted in dramatically reduced mouse T-cell proliferation. These results may indicate that the altered toxin may compete with wild type toxin for TCR binding.
- 40 SEA WT (10 LD50), site-specific SEA mutants (10 µg/mouse each) or LPS (150 µg/mice) injected alone were nonlethal to mice (Table 5). However, combining

LPS with either WT SEA or mutant K14E resulted in 100% lethality. For those mice receiving both LPS and WT or K14E SEA, 80% were dead by 24 h and 100% by 48 h. In contrast, 100% of Y92A and 80% of Y64A injected mice (coadministered with LPS) survived. The average time to death for the 20% of mice that did not survive Y64A injection occurred at 48 to 72 h. These *in vivo* data correlated well with the results obtained with the lymphocyte cultures. It was concluded that the observed attenuation of toxicity in mice was a direct result of the reduced T-cell proliferation.

Table 5. Biologic effect of wild type (WT) staphylococcal enterotoxin A (SEA) and SEA mutants.

Protein	No. live/total
WT	0/10
K14E	0/10
Y64A	8/10
Y92A	10/10

NOTE. Mice were given 10 LD<sub>50</sub> (10ug) of WT or mutant SEA. Lipopolysaccharide (150 ug/mouse) was injected 3 h later.

Having established that attenuation of receptor binding resulted in reduced toxicity, we next examined the immunogenicity of the SEA mutants. Mice were immunized with WT or mutant SEA. Control mice received adjuvant only or were left untreated. One week before challenge with WT SEA, mice were bled and serum antibody titers were determined for each group (Table 6). Mice immunized with the 2 µg of Y64A or Y92A had serum antibody titers of 1:5000 and 1:1000, respectively. Immunization with 2 µg of WT SEA or

control mutant resulted in titers of 1:5,000 and 1:10,000, respectively. The highest immunizing dose (10 µg/mouse) was most effective for all animals, resulting in antibody titers which were greater than 1:10,000. All mice were challenged with 10 LD50 of WT SEA (potentiated with LPS). The survival data correlated well with the levels of serum antibodies in immunized mice. All mice that were vaccinated with 10 µg of Y64A or Y92A, survived the lethal challenge dose of WT SEA. Slightly less protection was afforded by the lower vaccination dose of mutant Y64A or Y92A. All mice immunized with both doses of WT SEA survived the lethal challenge with WT potentiated with LPS. Mice immunized with mutant K14E exhibited survivals of 100% and 80% for high and low vaccination doses, respectively. All nonimmunized or control mice that were vaccinated with adjuvant alone died when challenged with WT SEA and a potentiating dose of LPS.

Table 6. Mice immunized with attenuated forms of staphylococcal enterotoxin A (SEA) produce high titers of neutralizing antibody.

Immunizing agent	Dose (ug/mouse)	Anti-SEA antibody titer*	No. live/total
WT	2	10,000-50,000	10/10
	10	10,000-50,000	10/10
K14E	2	5,000-10,000	8/10
	10	10,000-50,000	10/10
Y64A	2	5,000-10,000	6/10
	10	10,000-50,000	10/10
Y92A	2	1,000-5,000	2/10
	10	10,000-50,000	10/10
Adjuvant		50-100	0/10

NOTE. Mice were given 10 LD<sub>50</sub> of wild type (WT) SEA challenge followed by potentiating dose of lipopolysaccharide (150 ug/mouse) 3 h later.

- ! \*Reciprocal of serum dilution resulting in optical  
5 density reading four times above negative controls (wells containing either no SEA or no primary antibody).

#### EXAMPLE 8

##### Immune recognition of SAg mutants.

- 10 Bacterial SAGs induce clonal anergy of specific subsets of T cells in mice. It was possible that the loss of sensitivity to WT SEA among the mice vaccinated with the attenuated mutant forms represented a state of specific non-responsiveness  
15 instead of specific immunity. To address this issue, lymphocyte responses to SEA WT were measured with splenic mononuclear cells collected 2 weeks after the third immunization. As expected, lymphocytes from mice that were immunized with WT SEA or control SEA  
20 mutant showed little to no proliferation when incubated with the WT SAg. In contrast, lymphocytes obtained from control mice or those immunized with either Y64A or Y92A all responded vigorously to the WT SEA (Fig. 5). The TCRs used by T cells from the SEA-  
25 vaccinated mice were then characterized by flow cytometry. T cells from immunized or control mice were incubated with WT SEA in culture for 7 days, followed by a 5 day expansion in IL-2 containing medium. Distinct populations of activated TCR V $\beta$ 11  
30 positive cells were observed with T cells from mice immunized with Y92A and Y64A, representing 48% and 40% of T cells, respectively. However, V $\beta$ 11 expressing cells obtained from SEA WT or K14E immunized mice were about 1% and 6% of the total T-cell population,  
35 respectively, suggesting that this subset was

nonresponsive to restimulation with the WT SAg. T cells bearing VB 17a, 3, 7, and 10b were unchanged for all mice. It was apparent that T-cell responses to both the TCR and MHC class II binding-attenuated SEA mutants were similar to each other, but differed from responses to control or WT molecules. These results suggested that an alternative, perhaps conventional antigen processing mechanism was functioning in presentation of the SAg mutants Y64A and Y92A.

10

#### EXAMPLE 9

##### Rhesus monkey immunizations with monovalent vaccines.

The SEA vaccine L48R, Y89A, D70R (A489270) and SEB vaccine Y89A, Y94A, L45R (B899445) were used to immunize rhesus monkeys. The animals received a total of three i.m. injections (10-20 µg/animal), given at monthly intervals. Rhesus monkeys that were injected with these vaccines had no detectable increase of serum cytokines and no apparent toxicity. The serological response of animals vaccinated with three doses of formalin-treated SEB toxoid (100 µg/injection) gave results comparable to one or two injections with B899445 (Table 7), suggesting that the recombinant vaccines were very immunogenic. Immunized rhesus monkeys survived a lethal challenge with >10 LD50 of wild-type SEB (Table 7, 8). Collectively, these results suggest that the engineered SEB vaccine is safe, highly antigenic and effective at protecting the immunized individual from lethal aerosol exposure to SEB.

35

Table 7. Rhesus monkey antibody responses to vaccine B899445; One injection of B899445 outperforms three injections of SEB toxoid

	Vaccine <sup>1</sup> /animal #	Antibody response <sup>2</sup>	%Inhibition of T-cell response <sup>3</sup>	Survival SEB >20 x LD50 challenge <sup>4</sup>
5				
10	preimmune sera /pooled	0.161	5	dead
	toxoid/1	0.839	0	dead
15	toxoid/2	0.893	34	live
	toxoid/3	1.308	57	live
	toxoid/4	1.447	55	live
20	B899445/1	1.788	69	live
	B899445/2	0.78	49	live

25

<sup>1</sup>Rhesus monkeys were immunized with one dose (20 µg injection) of B899445 vaccine or three doses of formalin-treated SEB toxoid (100 µg/injection) one month apart; both used Alum adjuvants.

30

<sup>2</sup>Sera were collected one month after the final injection. Antibody responses were determined by ELISA and the results are shown as mean optical densities of triplicate wells (± SEM).

35

<sup>3</sup>Rhesus monkey T cells, obtained from an untreated animal, were preincubated with diluted (1:70) serum from immunized monkeys and then cultured with wild type SEB. Data are shown as % of T cell responses, where serum of rhesus monkey injected with adjuvant only represented the 100% of response to wild type SEB.

40

<sup>4</sup>Rhesus monkeys were challenged by aerosol exposure and monitored for four days.

45

50

Table 8. Engineered staphylococcal enterotoxin B vaccine efficacy in rhesus monkeys

5	Treatment <sup>1</sup>	Antibody titer <sup>2</sup>	Immune protection <sup>3</sup>
	Vaccine with adjuvant	>10,000	100%
10	Adjuvant only	<50	0%

15 <sup>1</sup>Rhesus monkeys (n=10) were injected i.m. with 10 µg of SEB vaccine with Alhydrogel adjuvant. A total of 3 immunizations, 1 month apart were given. Controls (n=2) received only Alhydrogel.

20 <sup>2</sup>Serum dilution resulting in optical density readings of four times above the negative control, consisting of no SEB or serum added to the wells.

<sup>3</sup>Immunized and control rhesus monkeys were challenged with >10 LD50 of wild-type staphylococcal enterotoxin B as an aerosol.

25 Serum from monkeys that were immunized with the genetically attenuated vaccine inhibited T-lymphocyte responses to wild type SEB (Table 7) similarly or better than monkeys that received the SEB toxoid. Collectively, these results suggest that the  
30 recombinant SAg vaccines are safe, highly antigenic, and induce protective immunity.

Serum from B899445 immunized rhesus monkeys blocked human lymphocyte responses to wild-type superantigen when tested in ex vivo cultures (Table  
35 7). These data again showed that the second and third injections of vaccine were approximately equivalent in stimulating neutralizing antibody responses. Normal T-cell responses to several superantigens, including the wild-type protein, were observed in immunized  
40 animals, indicating that no specific or generalized anergy occurred (Fig. 6).

**EXAMPLE 10****A. Multivalent superantigen vaccines: Rhesus monkey immunizations.**

1 Rhesus monkeys were immunized with a combined  
5 vaccine consisting of B899445 and A489270. Following  
the third injection, antibody recognition of wild-type  
bacterial superantigens was examined (Fig.7). High  
titers of anti-SEB, SEC1 and SEA antibodies were  
evident.

10

**B. Mouse immunizations.**

Mice (BALB/c) were immunized with a combined  
vaccine consisting of SEA, SEB, SEC1 and TSST-1 (all  
wild-type). The antibody responses against each  
15 individual superantigen were assessed (Table 9).  
Antibodies were induced against each of the component  
antigens, providing sufficient levels to protect the  
mice from a lethal challenge of superantigen,  
potentiated with LPS. Although not shown in the  
20 Table, antibody responses against SPE-A were also  
observed. Mice were also immunized with individual  
superantigens and antibody responses against other  
superantigens were measured (Table 10). Each  
individual immunogen induced partial or complete  
25 protective antibody responses against all other  
superantigens tested.

30



TABLE 9. Superantigen cross-reactivity of antibodies from mice immunized with individual bacterial superantigens

5	Immunizing <sup>1</sup> Toxin	Challenging <sup>2</sup> Toxin	ELISA <sup>3</sup> Titer	Neutralizing <sup>4</sup> Antibody
	SEA	SEA	>1/25,000	100%
	SEA	SEB	>1/25,000	100%
10	SEA	SEC1	>1/25,000	100%
	SEA	TSST1	>1/10,000	100%
	SEB	SEB	>1/25,000	100%
	SEB	SEA	>1/10,000	100%
	SEB	SEC1	>1/2,500	100%
15	SEB	TSST1	>1/10,000	100%
	SEC1	SEC1	>1/10,000	100%
	SEC1	SEA	>1/10,000	100%
	SEC1	SEB	>1/25,000	100%
	SEC1	TSST1	>1/10,000	100%
20	TSST1	TSST1	<1/10,000	100%
	TSST1	SEA	<1/1,000	50%
	TSST1	SEB	<1/1,000	40%
	TSST1	SEC1	<1/1,000	40%

25 <sup>1</sup>Three injections with 20 ug of antigen (BALB/c mice).

<sup>2</sup>LPS-potentiated challenge with 10 LD<sub>50</sub>s of superantigen.

<sup>3</sup>ELISA antibody response against an individual superantigen.

30 <sup>4</sup>Percent mice surviving an LPS-potentiated challenge (n=10).

Table 10. Multivalent superantigen vaccine. Mouse immune responses.

	Immunizing toxin <sup>1</sup>	Challenging toxin <sup>1</sup>	Antibody Titer <sup>2</sup>	% survival
5	SE-A, B, C1, TSST-1	all	N/A	100%
10	" "	SEA	>25,000	100%
	" "	SEB	>25,000	100%
	" "	SEC1	>25,000	100%
15	" "	TSST-1	>6,400	100%

20 <sup>1</sup>Total of three injections, two weeks apart, in RIBI adjuvant.

<sup>2</sup>>10 X LD50, potentiated with *E. coli* lipopolysaccharide.

<sup>3</sup>Measured by ELISA.

25

#### EXAMPLE 11

##### Design of altered TSST-1 toxin vaccine, TST30.

A comprehensive study of the relationships of TSST-1 protein structure to receptor binding were undertaken to provide insight into the design of the vaccine TST30. We have discovered that TSST-1 interactions with the human MHC class II receptor, HLA-DR, are relatively weak and can be disrupted by altering only a single critical amino acid residue of the toxin. Site-directed mutagenesis of a gene encoding the toxin and expression of the new protein product in *E. coli* were then used to test the design of the vaccine. The TSST-1 gene used was contained within a fragment of DNA isolated by BglI restriction enzyme digestion of the gene isolated from a toxigenic strain of *Staphylococcus aureus* (AB259; Kreiswirth and Novick (1987) *Mol. Gen. Genet.* 208, 84-87). The sequence of this gene is identical to all currently

known TSST-1 isolates of human origin. The wild-type TSST-1 gene can be readily cloned from a number of clinical *S. aureus* isolates. The DNA fragment containing the TSST-1 gene was isolated by agarose gel electrophoresis and ligated into the prokaryotic expression vector pSE380 (Invitrogen Corp.). The DNA clone consisted of sequences encoding the leader peptide and the full length of the mature TSST-1 protein. This engineered vaccine is currently being evaluated to determine mouse and human T-cell reactivities in vitro, and lethality in mice. The TST30 vaccine consists of the following mutation introduced into the toxin molecule: leucine at amino acid residue 30 changed to arginine. Two other mutations, namely Asp27 to Ala and Ile46 to Ala have also been designed. The final vaccine may incorporate one or both of these additional mutations.

The binding interface between TSST-1 and HLA-DR consists of a large relatively flat surface located in the N-terminal domain. Leucine 30 protrudes from a reverse turn on the surface of TSST-1 and forms the major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 30 in TSST-1 to the charged amino acid side chain of arginine is predicted to disrupt this major contact with the receptor molecule, resulting in a significant reduction in DR1 binding. This mutant molecule should therefore have lost the toxin attributes of the wild-type molecule.

TST30 was expressed as a recombinant protein in *E. coli*, as either a periplasmically secreted protein or as a cytoplasmic product. Purification was achieved by immunoaffinity chromatography or preparative isoelectric focusing after an initial ion-exchange CM-Sepharose enrichment step. The method of

- purification was not critical to the performance of the vaccine. Lipopolysaccharide contaminants, resulting from expression in a Gram-negative bacterium, were readily removed (as determined by
- 5 limulus assay) using a variety of standard methods. The final purified vaccine is not toxic to mice at levels equivalent to 10 LD<sub>50</sub> of the native TSST-1. No indicators of toxicity were found in surrogate assays of human T-cell stimulation.
- 10 Conclusive vaccine studies demonstrating that TST30 is highly antigenic and induces protective immunity are in progress in a mouse animal model. Mouse lethality is achieved at less than 1 ug/animal when a potentiating signal like lipopolysaccharide
- 15 from Gram-negative bacteria (LPS) is provided. When coadministered with LPS, wild-type TSST-1 is 100% lethal to mice (10 LD<sub>50</sub>). Mice receive three injections (two weeks between injections) of 20 ug/mouse in alhydrogel and protection against the
- 20 lethal effects of 10 LD<sub>50</sub> of TSST-1 are assessed.

#### EXAMPLE 12

##### Design of altered SPEA toxin vaccine, SPEa42

- The SPEa interactions with human MHC class II
- 25 receptor, HLA-DR, are relatively weak and can be disrupted by altering only a single critical amino acid residue of the toxin. Site-directed mutagenesis of a gene encoding the toxin and expression of the new protein product in E.coli were then used to test the
- 30 design of the vaccine. The SPEa gene used was clone from a SPEa-toxigenic strain of *Streptococcus* by using specific DNA oligonucleotide primers and the polymerase chain reaction method. The sequence of this gene is identical to SPEa isolates of human
- 35 origin known within the public domain. The DNA

fragment containing the SPEa gene was isolated by agarose gel electrophoresis and ligated into a prokaryotic expression vector (pETx or pSE380). The DNA clone consisted of sequences encoding the leader peptide and the full length of the mature SPEa protein or SPEa42 without a leader sequence. We recognize that there are additional ways to express or produce the mature SPEa vaccine. The SPEa vaccine consists of the following mutation introduced into the toxin molecule: leucine at amino acid residue 42 changed to arginine.

The binding interface between SPEa and HLA-DR is predicted to consist of contacts located in the N-terminal domain that are conserved with other bacterial superantigens. Leucine 42 of SPEa is predicted to protrude from a reverse turn on the surface of SPEa and form a major hydrophobic contact with the HLA-DR receptor molecule. Mutation of the single residue leucine 42 in SPEa to the charged amino acid side chain of arginine is predicted to disrupt this major contact with the receptor molecule, resulting in a significant reduction in DR1 binding. This mutant molecule should therefore have lost the toxin attributes of the wild-type molecule.

SPEa42 was expressed as a recombinant protein in E.coli, as either a periplasmically secreted protein or as a cytoplasmic product. Purification was achieved by immunoaffinity chromatography or preparative isoelectric focusing after an initial ion-exchange CM-Sepharose enrichment step. The method of purification was not critical to the performance of the vaccine. Lipopolysaccharide contaminants, resulting from expression in a Gram-negative bacterium, were readily removed (as determined by limulus assay) using a variety of standard methods.

The final purified vaccine is not toxic to mice at levels equivalent to 10 LD<sub>50</sub> of the native TSST-1. No indicators of toxicity were found in surrogate assays of human T-cell stimulation.

- 5       Conclusive vaccine studies demonstrating that SPEa42 is highly antigenic and induces protective immunity are in progress in a mouse animal model. Mouse lethality is achieved at less than 1 ug/animal when a potentiating signal like lipopolysaccharide  
10   from Gram-negative bacteria (LPS) is provided. When coadministered with LPS, wild-type SPEa is 100% lethal to mice (10 LD<sub>50</sub>). Mice receive three injections (two weeks between injections) of 20 ug/mouse in alhydrogel and protection against the lethal effects of 10 LD<sub>50</sub> of  
15   SPEa are assessed

### EXAMPLE 13

#### Design of altered superantigen toxin vaccine.

##### SEC45

- 20       For Staphylococcal enterotoxin C1 (SEC1), the leucine at position 45 was changed to lysine (SEC45). This mutation is anticipated to prevent SEC1 from interacting with the MHC class II receptor by sterically blocking the hydrophobic loop (centered  
25   around leucine 45) from binding to the alpha chain of the receptor. SEC1 is more closely homologous to SEB than SEA or the other superantigen toxins. The presence of zinc in SEC1 may impart additional binding characteristics that allow, in some cases, this  
30   superantigen toxin to bind to T-cell antigen receptors without the required MHC class II molecule interactions. To circumvent the binding to T-cell antigen receptors, mutations of SEC1 residues N23

(changed to alanine), V91 (changed to lysine) are being performed.

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30

## SEQUENCE LISTING

- (1) GENERAL INFORMATION:
- 5 (i) APPLICANT: Robert G. Ulrich,  
Mark A. Olson  
Sina Bavari
- (ii) TITLE OF INVENTION: Bacterial Superantigen  
10 Vaccines
- (iii) NUMBER OF SEQUENCES:16
- (iv) CORRESPONDENCE ADDRESS:
- 15 (A) ADDRESSEE: John Moran  
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(C) CITY: FORT DETRICK  
(D) STATE: MARYLAND  
20 (E) COUNTRY: USA  
(F) ZIP: 21702-5012
- (v) COMPUTER READABLE FORM:
- 25 (A) MEDIUM TYPE: Floppy disk  
(B) COMPUTER: Apple Macintosh  
(C) OPERATING SYSTEM: Macintosh 7.5  
(D) SOFTWARE: Microsoft Word 6.0
- (vi) CURRENT APPLICATION DATA:
- 30 (A) APPLICATION NUMBER: 08/882,431  
(B) FILING DATE: June 25, 1997  
(C) CLASSIFICATION:
- (vii) PRIOR APPLICATION DATA:
- 35 (A) APPLICATION NUMBER:  
(B) FILING DATE:
- (viii) ATTORNEY/AGENT INFORMATION:
- 40 (A) NAME: Moran, John  
(B) REGISTRATION NUMBER: 26,313  
(C) REFERENCE/DOCKET NUMBER:
- (ix) TELECOMMUNICATION INFORMATION
- 45 (A) TELEPHONE: (301) 619-2065  
(B) TELEFAX: (301) 619-7714
- (2) INFORMATION FOR SEQUENCE ID NO:1:
- (i) SEQUENCE CHARACTERISTICS:
- 50 (A) LENGTH: 830  
(B) TYPE: Nucleic Acid



(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) Molecule type: DNA

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 GAATTGCAGG GAACAGCTTT AGGCAATCTT AAACAAATCT 160  
 15 ATTATTACAA TGAAAAAGCT AAAACTGAAA ATAAAGAGAG 200  
 TCACGATCAA TTTCGACAGC ATACTATATT GTTTAAAGGC 240  
 TTTTTTACAG ATCATTCGTG GTATAACGAT TTATTAGTAC 280  
 20 GTTTTGATTG AAAGGATATT GTTGATAAAT ATAAAGGGAA 320  
 AAAAGTAGAC TTGTATGGTG CTTATGCTGG TTATCAATGT 360  
 25 GCGGGTGGTA CACCAAACAA AACAGCTTGT ATGTATGGTG 400  
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 AAAAGTGCCG ATCAATTTAT GGCTAGACGG TAAACAAAAT 480  
 30 ACAGTACCTT TGGAAACGGT TAAAACGAAT AAGAAAAATG 520  
 TAACTGTTCA GGAGTTGGAT CTTCAAGCAA GACGTTATTT 560  
 35 ACAGGAAAAA TATAATTTAT ATAACCTCTGA TGTTTTTGAT 600  
 GGAAGGTTT AGAGGGGATT AATCGTGTTT CATACTTCTA 640  
 CAGAACCTTC GGTTAATTAC GATTTATTTG GTGCTCAAGG 680  
 40 ACAGTATTCA AATACACTAT TAAGAATATA TAGAGATAAT 720  
 AAAACGATTA ACTCTGAAAA CATGCATATT GATATATATT 760  
 TATATACAAG TTAAACATGG TAGTTTTGAC CAACGTAATG 800  
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(3) INFORMATION FOR SEQUENCE ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

50 (A) LENGTH: 257  
 (B) TYPE: Amino Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

(ii) Molecule type: Peptide

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 Leu Val Asn Gly Ser Glu Lys Ser Glu Glu  
 25 30  
 15 Ile Asn Glu Lys Asp Leu Arg Lys Lys Ser  
 35 40  
 Glu Lys Gln Gly Thr Ala Leu Gly Asn Leu  
 45 50  
 20 Lys Gln Ile Tyr Tyr Tyr Asn Glu Lys Ala  
 55 60  
 25 Lys Thr Glu Asn Lys Glu Ser His Asp Gln  
 65 70  
 Phe Arg Gln His Thr Ile Leu Phe Lys Gly  
 75 80  
 30 Phe Phe Thr Asp His Ser Trp Tyr Asn Asp  
 85 90  
 Leu Leu Val Arg Phe Asp Ser Lys Asp Ile  
 95 100  
 35 Val Asp Lys Tyr Lys Gly Lys Lys Val Asp  
 105 110  
 40 Leu Tyr Gly Ala Tyr Ala Gly Tyr Gln Cys  
 115 120  
 Ala Gly Gly Thr Phe Asn Lys Thr Ala Cys  
 125 130  
 45 Met Tyr Gly Gly Val Thr Leu His Asp Asn  
 135 140  
 Asn Arg Leu Thr Glu Glu Lys Lys Val Pro  
 145 150  
 50 Ile Asn Leu Trp Leu Asp Gly Lys Gln Asn

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	Thr Val Pro Leu Glu Thr Val Lys Thr Asn			
		165		170
5	Lys Lys Asn Val Thr Val Gln Glu Leu Asp			
		175		180
	Leu Gln Ala Arg Arg Tyr Leu Gln Glu Lys			
10		185		190
	Tyr Asn Leu Tyr Asn Ser Asp Val Phe Asp			
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15	Gly Lys Val Gln Arg Gly Leu Ile Val Phe			
		205		210
	His Thr Ser Thr Glu Pro Ser Val Asn Tyr			
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	Asp Leu Phe Gly Ala Gln Gly Gln Tyr Ser			
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	Asn Thr Leu leu Arg Ile Tyr Arg Asp Asn			
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	Lys Thr Ile asn Ser Glu Asn Met His Ile			
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	(A) LENGTH: 757	
	(B) TYPE: Nucleic Acid	
	(C) STRANDEDNESS: Unknown	
	(D) TOPOLOGY: Unknown	
	(ii) Molecule type: DNA	
40	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:	
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 10 ACAAATACA GTACCTTTGG AAACGGTTAA AACGAATAAG 440  
 AAAAATGTAA CTGTTCAAGG GTTGGATCTT CAAGCAAGAC 480  
 15 GTTATTTACA GGAAAAATAT AATTATATA ACTCTGATGT 520  
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 20 CTCAGGACA GTATTCAAAT AACTATTAA GAATATATAG 640  
 AGATAATAAA ACGATTAAC CTGAAAACAT GCATATTGAT 680  
 25 ATATATTTAT ATACAAGTTA AACATGGTAG TTTTGACCAA 720  
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## (5) INFORMATION FOR SEQUENCE ID NO:4:

## 30 (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH:233

(B) TYPE: Amino Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

## 35 (ii) Molecule type: Peptide

.(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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 5 10  
 40 Asp Leu Arg Lys Lys Ser Glu Lys Gln Gly  
 15 20  
 45 Thr Ala Leu Gly Asn Leu Lys Gln Ile Tyr  
 25 30  
 Tyr Tyr Asn Glu Lys Ala Lys Thr Glu Asn  
 35 40  
 50 Lys Glu Ser His Asp Gln Phe Arg Gln His  
 45 50

	Thr Ile Leu Phe Lys Gly Phe Phe Thr Asp	55	60
5	His Ser Trp Tyr Asn Asp Leu Leu Val Arg	65	70
	Phe Asp Ser Lys Asp Ile Val Asp Lys Tyr	75	80
10	Lys Gly Lys Lys Val Asp Leu Tyr Gly Ala	85	90
	Tyr Ala Gly Tyr Gln Cys Ala Gly Gly Thr	95	100
15	Pro Asn Lys Thr Ala Cys Met Tyr Gly Gly	105	110
	Val Thr Leu His Asp Asn Asn Arg Leu Thr	115	120
	Glu Glu Lys Lys Val Pro Ile Asn Leu Trp	125	130
25	Leu Asp Gly Lys Gln Asn Thr Val Pro Leu	135	140
	Glu Thr Val Lys Thr Asn Lys Lys Asn Val	145	150
30	Thr Val Gln Glu Leu Asp Lys Gln Ala Arg	155	160
	Arg Tyr Leu Gln Glu Lys Tyr Asn Leu Tyr	165	170
35	Asn Ser Asp Val Phe Asp Gly Lys Val Ala	175	180
40	Arg Gly Leu Ile Val Phe His Thr Ser Thr	185	190
	Glu Pro Ser Val Asn Tyr Asp Leu Phe Gly	195	200
45	Ala Gln Gly Gln Tyr Ser Asn Thr Leu Leu	205	210
	Arg Ile Tyr Arg Asp Asn Lys Thr Ile Asn	215	220

Ser Glu Asn Met His Ile Asp Ile Tyr Leu  
 225 230

Tyr Thr Ser

5

(6) INFORMATION FOR SEQUENCE ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- 10 (A) LENGTH: 1712  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Unknown  
 (D) TOPOLOGY: Unknown

(ii) Molecule type: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

15 GAACTAGGTA GAAAAATAAT TATGAGAAAA CACTATGTTG 40  
 TTAAAGATGT TTTCGTATAT AAGTTTAGGT GATGTATAGT 80  
 20 TACTTAATTT TAAAAGCATA ACTTAATTAA TATAAATAAC 120  
 ATGAGATTAT TAAATATAAT TAAGTTTCTT TTAATGTTTT 160  
 25 TTTAATTGAA TATTTAAGAT TATAACATAT ATTTAAAGTG 200  
 TATCTAGATA CTTTTTGGGA ATGTTGGATA AAGGAGATAA 240  
 AAAATGTATA AGAGATTATT TATTTACAT GTAAATTTGA 280  
 30 TATTCGCACT GATATTAGTT ATTTCTACAC CCAACGTTTT 320  
 AGCAGAGAGT CAACCAGATC CTAAACCAGA TGAGTTGCAC 360  
 35 AAATCGAGTA AATTCACCTGG TTTGATGGAA GATATGAAAG 400  
 TTTTGTATGA TGATAATCAT GTATCAGCAA TAAACGTTAA 440  
 ATCTATAGAT CAATTTCTAT ACTTTGACTT AATATATTCT 480  
 40 ATTAAGGACA CTAAGTTAGG GGATTATGAT AATGTTTCGAG 520  
 TCGAATTTAA AAACAAAGAT TTAGCTGATA AATACAAAGA 560  
 45 TAAATACGTA GATGTGTTTG GAGCTAATTA TTATTATCAA 600  
 TGTTATTTTT CTAAAAAAC GAATGATATT AATTCGCATC 640  
 AAACGACAA ACGAAAAACT TGTATGTATG GTGGTGTAAAC 680  
 50 TGAGCATAAT GGAAACCAAT TAGATAAATA TAGAAGTATT 720

	ACTGTTCTGGG TATTTGAAGA TGGTAAAAAT TTATTATCTT	760
	TTGACGTACA AACTAATAAG AAAAAGGTGA CTGCTCAAGA	800
5	ATTAGATTAC CTAACCTCGTC ACTATTTGGT GAAAAATAAA	840
	AAACTCTATG AATTTAACAA CTCGCCCTAT GAAACGGGAT	880
10	ATATTAAATT TATAGAAAAT GAGAATAGCT TTTGGTATGA	920
	CATGATGCCT GCACCAGGAG ATAAATTGTC CCAATCTAAA	960
	TATTTAATGA TGTACAATGA CAATAAAATG GTTGATTCTA	1000
15	AAGATGTGAA GATTGAAGTT TATCTTACGA CAAAGAAAAA	1040
	GTGAAATTAT ATTTTAGAAA AGTAAATATG AAGAGTTAGT	1080
20	AATTAAGGCA GGCACCTATA GAGTACCTGC CTTTCTAAT	1120
	ATTATTTAGT TATAGTTATT TTTGTTATAT CTCTCTGATT	1160
	TAGCATTAAC CCCTTGTTGC CATTATAGTT TTCACCAACT	1200
25	TTAGCTGAAA TTGGGGGATC ATTTTATCT TTAATATGGA	1240
	TAGTTACTGT GTCGCCGTTT TTAACGATT GTTCTCTTTT	1280
30	TAATTTGTCA GTTAATTTTT TCCATGCATC ATTTGCGTCA	1320
	AACCTATTTT CATTGGGATT TATCTTGAC AAATCAATTC	1360
	TTTTAACACT ATCGGTATTA ATCGGCTTGT TATTAAAATT	1400
35	ACTAAGTTCA TCTAAATCAG CTGTACCCGT AATACTACTT	1440
	TCGCCACCAT TATTTAAATT GTACGTAACA CCAACTGTCT	1480
40	CATTTGCTGT TTTATCGATA ATATTGCTT CTTTCAAAGC	1520
	ATCTCTTACA TTTTCCATA AGTCTCTATC TGTATTTCAT	1560
	GAAGCCTTTG CAACGTTATT AATACCATTA TAATTTGAAG	1600
45	AAGAATGAAA ACCTGAACCT ACTGTTGTGA AACTAAAGC	1640
	ACTTGCTATC AATGTTCTTG TTAATAGTTT TTTATTCATT	1680
50	TTATTTCTC CTATAACTTA TTTGCAATCG AT	1712

## (7) INFORMATION FOR SEQUENCE ID NO:6:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 265  
 (B) TYPE: Amino Acid  
 (C) STRANDEDNESS: Unknown  
 (D) TOPOLOGY: Unknown

## (ii) Molecule type: Peptide

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

10 Met Tyr Lys Arg Leu Phe Ile Ser His Val  
                                   5                                  10

Ile Leu Ile Phe Ala Leu Ile Leu Val Ile  
                                   15                                  20

15 Ser Thr Pro Asn Val Leu Ala Glu Ser Gln  
                                   25                                  30

20 Pro Asp Pro Lys Pro Asp Glu Leu His Lys  
                                   35                                  40

Ser Ser Lys Phe Thr Gly Leu Met Glu Asp  
                                   45                                  50

25 Met Lys Val Leu Tyr Asp Asp Asn His Val  
                                   55                                  60

Ser Ala Ile Asn Val Lys Ser Ile Asp Gln  
                                   65                                  70

30 Phe Leu Tyr Phe Asp Leu Ile Tyr Ser Ile  
                                   75                                  80

35 Lys Asp Thr Lys Leu Gly Asp Tyr Asp Asn  
                                   85                                  90

Val Arg Val Glu Phe Lys Asn Lys Asp Leu  
                                   95                                  100

40 Ala Asp Lys Tyr Lys Asp Lys Tyr Val Asp  
                                   105                                  110

Val Phe Gly Ala Asn Tyr Tyr Tyr Gln Cys  
                                   115                                  120

45 Tyr Phe Ser Lys Lys Thr Asn Asp Ile Asn  
                                   125                                  130

50 Ser His Gln Thr Asp Lys Arg Lys Thr Cys  
                                   135                                  140



	Met Tyr Gly Gly Val Thr Glu His Asn Gly	145	150
5	Asn Gln Leu Asp Lys Tyr Arg Ser Ile Thr	155	160
	Val Arg Val Phe Glu Asp Gly Lys Asn Leu	165	170
10	Leu Ser Phe Asp Val Gln Tyr Asn Lys Lys	175	180
	Lys Val Thr Ala Gln Glu Leu Asp Tyr Leu	185	190
15	Thr Arg His Tyr Leu Val Lys Asn Lys Lys	195	200
	Leu Tyr Glu Phe Asn Asn Ser Pro Tyr Glu	205	210
20	Thr Gly Tyr Ile Lys Phe Ile Glu Asn Gln	215	220
	Asn Phe Trp Tyr Asp Met Met Pro Ala Pro	225	230
25	Gly Asp Lys Phe Ala Gln Ser Lys Tyr Leu	235	240
30	Met Met Tyr Asn Asp Asn Lys Met Val Asp	245	250
	Ser Lys Asp Val Lys Leu Glu Val Tyr Leu	255	260
35	Thr Thr Lys Lys Lys	265	

40 (8) INFORMATION FOR SEQUENCE ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 1712

(B) TYPE: Nucleic Acid

(C) STRANDEDNESS: Unknown

(D) TOPOLOGY: Unknown

45

(ii) Molecule type: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

50	GAAC TAGGTA GAAAAATAAT TATGAGAAAA CACTATGTTG	40
	TTAAAGATGT TTTCGTATAT AAGTTTAGGT GATGTATAGT	80

	TACTTAATTT TAAAAGCATA ACTTAATTAA TATAAATAAC	120
5	ATGAGATTAT TAAATATAAT TAAGTTTCTT TTAATGTTTT	160
	TTTAATTGAA TATTTAAGAT TATAACATAT ATTTAAAGTG	200
	TATCTAGATA CTTTTTGGGA ATGTTGGATA AAGGAGATAA	240
10	AAAATGTATA AGAGATTATT TATTTACAT GTAATTTTGA	280
	TATTCGCACT GATATTAGTT ATTTCTACAC CCAACGTTTT	320
15	AGCAGAGAGT CAACCAGATC CTAAACCAGA TGAGTTGCAC	360
	AAATCGAGTA AATTCACGGG TTTGATGGAA AATATGAAAG	400
	TTTGTATGA TGATAATCAT GTATCAGCAA TAAACGTTAA	440
20	ATCTATAGAT CAATTTGAT ACTTTGACTT AATATATTCT	480
	ATTAAGGACA CTAAGTTAGG GAATTATGAT AATGTTGAG	520
25	TCGAATTTAA AAACAAAGAT TTAGCTGATA AATACAAAGA	560
	TAAATACGTA GATGTGTTTG GAGCTAATGC TTATTATCAA	600
	TGTGCTTTTT CTAAAAAAC GAATGATATT AATTCGCATC	640
30	AAACTGACAA ACGAAAACT TGTATGTATG GTGGTGTAA	680
	TGAGCATAAT GGAAACCAAT TAGATAAATA TAGAAGTATT	720
35	ACTGTTCCGG TATTTGAAGA TGGTAAAAAT TTATTATCTT	760
	TTGACGTACA AACTAATAAG AAAAAGGTGA CTGCTCAAGA	800
	ATTAGATTAC CTAACGTC ACTATTTGGT GAAAAATAAA	840
40	AAACTCTATG AATTTAACAA CTCGCCCTTAT GAAACGGGAT	880
	ATATTAAATT TATAGAAAAT GAGAATAGCT TTTGGTATGA	920
45	CATGATGCCT GCACCAGGAG ATAAATTTGA CCAATCTAAA	960
	TATTTAATGA TGTACAATGA CAATAAAATG GTTGATTCTA	1000
	AAGATGTGAA GATTGAAGTT TATCTTACGA CAAAGAAAAA	1040
50	GTGAAATTAT ATTTTAGAAA AGTAAATATG AAGAGTTAGT	1080
	AATTAAGGCA GGCACCTATA GAGTACCTGC CTTTCTAAT	1120

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ATTATTTAGT TATAGTTATT TTTGTTATAT CTCTCTGATT 1160
TAGCATTAAAC CCCTTGTTGC CATTATAGTT TTCACCAACT 1200
5 TTAGCTGAAA TTGGGGGATC ATTTTATCT TTAATATGGA 1240
TAGTTACTGT GTCGCCGTTT TTAACGATTT GTTTCTCTTT 1280
10 TAATTTGTCA GTTAATTTTT TCCATGCATC ATTTGCGTCA 1320
AACCTATTTT CATTTGGATT TATTCTTGAC AAATCAATTC 1360
15 TTTTAACTACT ATCGGTATTA ATCGGCTTGT TATTAAAAAT 1400
ACTAAGTTCA TCTAAATCAG CTGTACCCGT AATACTACTT 1440
TCGCCACCAT TATTTAAATT GTACGTAACA CCAACTGTCT 1480
20 CATTTGCTGT TTTATCGATA ATATTGCTT CTTTCAAAGC 1520
ATCTCTTACA TTTTCCATA AGTCTCTATC TGTTATTTCA 1560
GAAGCCTTTG CAACGTTATT AATACCATTA TAATTTGAAG 1600
25 AAGAATGAAA ACCTGAACCT ACTGTTGTTA AACTAAAGC 1640
ACTTGCTATC AATGTTCTTG TTAATAGTTT TTTATTCATT 1680
30 TTATTTTCTC CTATAACTTA TTTGCAATCG AT 1712

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## (9) INFORMATION FOR SEQUENCE ID NO:8:

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35 (i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 265
    (B) TYPE: Amino Acid
    (C) STRANDEDNESS: Unknown
    (D) TOPOLOGY: Unknown
(ii) Molecule type: Peptide
40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:
    Met Tyr Lys Arg Leu Phe Ile Ser His Val
                                5          10
45 Ile Leu Ile Phe Ala Leu Ile Leu Val Ile
                                15          20
    Ser Thr Pro Asn Val Leu Ala Glu Ser Gln
                                25          30
50 Pro Asp Pro Lys Pro Asp Glu Leu His Lys

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					35						40
		Ser	Ser	Lys	Phe	Thr	Gly	Leu	Met	Glu	Asn
						45					50
5		Met	Lys	Val	Leu	Tyr	Asp	Asp	Asn	His	Val
						55					60
		Ser	Ala	Ile	Asn	Val	Lys	Ser	Ile	Asp	Gln
10						65					70
		Phe	Arg	Tyr	Phe	Asp	Leu	Ile	Tyr	Ser	Ile
						75					80
15		Lys	Asp	Thr	Lys	Leu	Gly	Asp	Tyr	Asp	Asn
						85					90
		Val	Arg	Val	Glu	Phe	Lys	Asn	Lys	Asp	Leu
20						95					100
		Ala	Asp	Lys	Tyr	Lys	Asp	Lys	Tyr	Val	Asp
						105					110
25		Val	Phe	Gly	Ala	Asn	Ala	Tyr	Tyr	Gln	Cys
						115					120
		Ala	Phe	Ser	Lys	Lys	Thr	Asn	Asp	Ile	Asn
						125					130
30		Ser	His	Gln	Thr	Asp	Lys	Arg	Lys	Thr	Cys
						135					140
		Met	Tyr	Gly	Gly	Val	Thr	Glu	His	Asn	Gly
35						145					150
		Asn	Gln	Leu	Asp	Lys	Tyr	Arg	Ser	Ile	Thr
						155					160
40		Val	Arg	Val	Phe	Glu	Asp	Gly	Lys	Asn	Leu
						165					170
		Leu	Ser	Phe	Asp	Val	Gln	Tyr	Asn	Lys	Lys
						175					180
45		Lys	Val	Thr	Ala	Gln	Glu	Leu	Asp	Tyr	Leu
						185					190
		Thr	Arg	His	Tyr	Leu	Val	Lys	Asn	Lys	Lys
50						195					200
		Leu	Tyr	Glu	Phe	Asn	Asn	Ser	Pro	Tyr	Glu
						205					210

Thr Gly Tyr Ile Lys Phe Ile Glu Asn Gln  
 215 220  
 5 Asn Phe Trp Tyr Asp Met Met Pro Ala Pro  
 225 230  
 Gly Asp Lys Phe Asp Gln Ser Lys Tyr Leu  
 235 240  
 10 Met Met Tyr Asn Asp Asn Lys Met Val Asp  
 245 250  
 15 Ser Lys Asp Val Lys Leu Glu Val Tyr Leu  
 255 260  
 Thr Thr Lys Lys Lys  
 265  
 20  
 (10) INFORMATION FOR SEQUENCE ID NO:9:  
 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 1388  
 (B) TYPE: Nucleic Acid  
 25 (C) STRANDEDNESS: Unknown  
 (D) TOPOLOGY: Unknown  
 (ii) Molecule type: DNA  
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:  
 30 ATGAGTCAAC CAGATCCTAA ACCAGATGAG TTGCACAAAT 40  
 CGAGTAAATT CACTGGTTTG ATGGAAAATA TGAAAGTTTT 80  
 GTATGATGAT AATCATGTAT CAGCAATAAA CGTTAAATCT 120  
 35 ATAGATCAAT TTCGATACTT TGACTTAATA TATTCTATTA 160  
 AGGACACTAA GTTAGGGAAT TATGATAATG TTCGAGTCGA 200  
 40 ATTTAAAAAC AAAGATTTAG CTGATAAATA CAAAGATAAA 240  
 TACGTAGATG TGTTTGGAGC TAATGCTTAT TATCAATGTG 280  
 CTTTTTCTAA AAAAACGAAT GATATTAATT CGCATCAAAC 320  
 45 TGACAAACGA AAAACTTGTA TGTATGGTGG TGTAAC TGAG 360  
 CATAATGGAA ACCAATTAGA TAAATATAGA AGTATTACTG 400  
 50 TTCGGGTATT TGAAGATGGT AAAAATTTAT TATCTTTTGA 440

	CGTACAACT AATAAGAAAA AGGTGACTGC TCAAGAATTA	480
	GATTACCTAA CTCGTCCTA TTTGGTGAAA AATAAAAAAC	520
5	TCTATGAATT TAACAACCTG CCTTATGAAA CGGGATATAT	560
	TAAATTTATA GAAAATGAGA ATAGCTTTTG GTATGACATG	600
10	ATGCCTGCAC CAGGAGATAA ATTTGACCAA TCTAAATATT	640
	TAATGATGTA CAATGACAAT AAAATGGTTG ATTCTAAAGA	680
	TGTGAAGATT GAAGTTTATC TTACGACAAA GAAAAAGTGA	720
15	AATTATATTT TAGAAAAGTA AATATGAAGA GTTAGTAATT	760
	AAGGCAGGCA CTTATAGAGT ACCTGCCTTT TCTAATATTA	800
20	TTTAGTTATA GTTATTTTTG TTATATCTCT CTGATTTAGC	840
	ATTAACCCCT TGTGCCATT ATAGTTTTTCAC CAACTTTAG	880
	CTGAAATTGG GGGATCATT TTATCTTTAC TATGGATAGT	920
25	TACTGTGTCG CCGTTTTTAA CGATTTGTTT CTCTTTTAAT	960
	TTGTCAGTTA ATTTTTTCCA TGCATCATTT GCGTCAAACC	1000
30	TATTTCCATT TGGATTTATT CTTGACAAAT CAATTCTTTT	1040
	AACACTATCG GTATTAATCG GCTTGTATT AAAATTACTA	1080
	AGTTCATCTA AATCAGCTGT ACCCGTAATA CTACTTTCGC	1120
35	CACCATTATT TAAATTGTAC GTAACACCAA CTGTCTCATT	1160
	TGCTGTTTTA TCGATAATAT TTGCTTCTTT CAAAGCATCT	1200
40	CTTACATTTT TCCATAAGTC TCTATCTGTT ATTTCAGAAG	1240
	CCTTGCAAC GTTATTAATA CCATTATAAT TTGAAGAAGA	1280
	ATGAAAACCT GAACCTACTG TTGTTAAAAC TAAAGCACTT	1320
45	GCTATCAATG TTCTTGTTAA TAGTTTTTTA TTCATTTTAT	1360
	TTTCTCCTAT AACTTATTTG CAATCGAT	1388

(11) INFORMATION FOR SEQUENCE ID NO:10:

50

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 239

(B) TYPE: Amino Acid  
 (C) STRANDEDNESS: Unknown  
 (D) TOPOLOGY: Unknown

(ii) Molecule type: Peptide

5 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

	Met Ser Gln Pro Asp Pro Lys Pro Asp Glu	
	5	10
10	Leu His Lys Ser Ser Lys Phe Thr Gly Lys	
	15	20
	Met Glu Asn Met Lys Val Leu Tyr Asp Asp	
	25	30
15	Asn His Val Ser Ala Ile Asn Val Lys Ser	
	35	40
	Ile Asp Gln Phe Arg Tyr Phe Asp Leu Ile	
20	45	50
	Tyr Ser Ile Lys Asp Thr Lys Leu Gly Asn	
	55	60
25	Tyr Asp Asn Val Arg Val Glu Phe Lys Asn	
	65	70
	Lys Asp Leu Ala Asp Lys Tyr Lys Asp Lys	
	75	80
30	Tyr Val Asp Val Phe Gly Ala Asn Ala Tyr	
	85	90
	Tyr Gln Cys Ala Phe Ser Lys Lys Thr Asn	
35	95	100
	Asp Ile Asn Ser His Gln Thr Asp Lys Arg	
	105	110
40	Lys Thr Cys Met Tyr Gly Gly Val Thr Glu	
	115	120
	His Asn Gly Asn Gln Leu Asp Lys Tyr Arg	
	125	130
45	Ser Ile Thr Val Arg Val Phe Glu Asp Gly	
	135	140
50	Lys Asn Leu Leu Ser Phe Asp Val Gln Thr	
	145	150

	Asn Lys Lys Lys Val Thr Ala Gln Glu Leu	155	160
5	Asp Tyr Leu Thr Arg His Tyr Leu Val Lys	165	170
	Asp Lys Lys Leu Tyr Glu Phe Asn Asn Ser	175	180
10	Pro Tyr Glu Thr Gly Tyr Ile Lys Phe Ile	185	190
	Glu Asn Glu Asn Ser Phe Trp Tyr Asp Met	195	200
15	Met Pro Ala Pro Gly Asp Lys Phe Asp Gln	205	210
	Ser Lys Tyr Leu Met Met Tyr Asn Asp Asn	215	220
20	Lys Met Val Asp Ser Lys Asp Val Lys Ile	225	230
25	Glu Val Tyr Leu Thr Thr Lys Lys Lys	235	

## (12) INFORMATION FOR SEQUENCE ID NO:11:

30	(i) SEQUENCE CHARACTERISTICS:	
	(A) LENGTH: 731	
	(B) TYPE: Nucleic Acid	
	(C) STRANDEDNESS: Unknown	
	(D) TOPOLOGY: Unknown	
35	(ii) Molecule type: DNA	
	(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:	
	TAAGGAGAAT TAAAAATGAA TAAAAAATTA CTAATGAATT	40
40	TTTTTATCGT AAGCCCTTTG TTGCTTGCGA CAACTGCTAC	80
	AGATTTTACC CCTGTTCCCT TATCATCTAA TCAAATAATC	120
45	AAAACTGCAA AAGCATCTAC AAACGATAAT ATAAAGGATT	160
	TGCTAGACTG GTATAGTAGT GGGTCTGACA CTTTTACAAA	200
	TAGTGAAGTT TTAGATAATT CCAGAGGATC TATGCGTATA	240
50	AAAAACACAG ATGGCAGCAT CAGCTTGATA ATTTTCCGA	280



5 GTCCTTATTA TAGCCCTGCT TTTACAAAAG GGGAAAAAGT 320  
 TGA CT TAAAC ACAAAAAGAA CTAAAAAAG CCAACATACT 360  
 AGCGAAGGAA CTTATATCCA TTTCCAAATA AGTGGCGTTA 400  
 CAAATACTGA AAAATTACCT ACTCCAATAG AACTACCTTT 440  
 10 AAAAGTTAAG GTTCATGGTA AAGATAGCCC CTTAAAGTAT 480  
 GGGCCAAAGT TCGATAAAAA ACAATTAGCT ATATCAACTT 520  
 TAGACTTTGA AATTCGTCAT CAGCTAACTC AAATACATGG 560  
 15 ATTATATCGT TCAAGCGATA AAACGGGTGG TTATTGAAAA 600  
 ATAACAATGA ATGACGGATC CACATATCAA AGTGATTTAT 640  
 CTAAAAAGTT TGAATACAAT ACTGAAAAAC CACCTATAAA 680  
 20 TATTGATGAA ATAAAAACTA TAGAAGCAGA AATTAATTAA 720  
 TTTACCACTT T 731

25 (13) INFORMATION FOR SEQUENCE ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233  
 (B) TYPE: Amino Acid  
 (C) STRANDEDNESS: Unknown  
 30 (D) TOPOLOGY: Unknown

(ii) Molecule type: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

35 Met Asn Lys Lys Leu Leu Met Asn Phe Phe  
 5 10  
 Ile Val Ser Pro Leu Leu Leu Ala Thr Thr  
 15 20  
 40 Ala Thr Asp Phe Thr Pro Val Pro Leu Ser  
 25 30  
 Ser Asn Gln Ile Ile Lys Thr Ala Lys Ala  
 35 40  
 45 Ser Thr Asn Asp Asn Ile Lys Asp Leu Leu  
 45 50  
 50 Asp Trp Tyr Ser Ser Gly Ser Asp Thr Phe  
 55 60

	Thr Asn Ser Glu Val Leu Asp Asn Ser Arg	65	70
5	Gly Ser Met Arg Ile Lys Asn Thr Asp Gly	75	80
	Ser Ile Ser Lys Ile Ile Phe Pro Ser Pro	85	90
10	Tyr Tyr Ser Pro Ala Phe Thr Lys Gly Glu	95	100
	Lys Val Asp Leu Asn Thr Lys Arg Thr Lys	105	110
15	Lys Ser Gln His Thr Ser Gly Thr Tyr Ile	115	120
	His Phe Gln Ile Ser Gly Val Thr Asn Thr	125	130
20	Glu Lys Leu Pro Thr Pro Ile Glu Leu Pro	135	140
	Leu Lys Val Lys Val His Gly Lys Asp Ser	145	150
	Pro Leu Lys Tyr Gly Pro Lys Phe Asp Lys	155	160
30	Lys Gln Leu Ala Ile Ser Thr Leu Asp Phe	165	170
	Glu Ile Arg His Gln Leu Thr Gln Ile His	175	180
35	Gly Leu Tyr Arg Ser Ser Asp Lys Thr Gly	185	190
	Gly Tyr Trp Lys Ile Thr Met Asn Asp Gly	195	200
	Ser Thr Tyr Gln Ser Asp Leu Ser Lys Lys	205	210
45	Phe Glu Tyr Asn Thr Glu Lys Pro Pro Ile	215	220
	Asn Ile Asp Glu Ile Lys Thr Ile Glu Ala	225	230
50	Glu Ile Asn		

## (14) INFORMATION FOR SEQUENCE ID NO:13:

## (i) SEQUENCE CHARACTERISTICS:

- 5 (A) LENGTH: 1095  
 (B) TYPE: Nucleic Acid  
 (C) STRANDEDNESS: Unknown  
 (D) TOPOLOGY: Unknown

## (ii) Molecule type: DNA

## 10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

	ATCATTAAAT ATAATTAATT TTCTTTTAAT ATTTTTTTTAA	40
	TTGAATATTT AAGATTATAA GATATATTTA AAGTGATCT	80
15	AGATACTTTT TGGGAATGTT GGATGAAGGA GATAAAAATG	120
	AATAAGAGTC GATTTATTTT ATGCGTAATT TTGATATTCG	160
20	CACTTATACT AGTTCCTTTT ACACCCAACG TATTAGCAGA	200
	GAGCCAACCA GACCCTACGC CAGATGAGTT GCACAAAGCG	240
	AGTAAATTCA CTGGTTTGAT GGAAAATATG AAAGTTTTAT	280
25	ATGATGATCA TTATGTATCA GCAACTAAAG TTAAGTCTGT	320
	AGATAAATTT AGGGCACATG ATTTAATTTA TAACATTAGT	360
30	GATAAAAAAC TGAAAAATTA TGACAAAGTG AAAACAGAGT	400
	TATTAAATGA AGGTTTAGCA AAGAAGTACA AAGATGAAGT	440
	AGTTGATGTG TATGGATCAA ATTACTATGT AACTGCTAT	480
35	TTTTCATCCA AAGATAATGT AGGTAAAGTT ACAGGTGGCA	520
	AACTTGTAT GTATGGAGGA ATAACAAAAC ATGAAGGAAA	560
40	CCACTTTGAT AATGGGAAC TACAAAATGT ACTTATAAGA	600
	GTTTATGAAA ATAAAAGAAA CACAATTTCT TTTGAAGTGC	640
	AACTGATAA GAAAAGTGTA ACAGCTCAAG AACTAGACAT	680
45	AAAAGCTAGG AATTTTTTTAA TTAATAAAAA AAATTTGTAT	720
	GAGTTTAAAC GTTCACCATA TGAAACAGGA TATATAAAAT	760
50	TTATTGAAAA TAACGGCAAT ACTTTTTGGT ATGATATGAT	800

	GCCTGCACCA	GGCGATAAGT	TTGACCAATC	TAAATATTTA	840
	ATGATGTACA	ACGACAATAA	AACGGTTGAT	TCTAAAAGTG	880
5	TGAAGATAGA	AGTCCACCTT	ACAACAAAGA	ATGGATAATG	920
	TTAATCCGAT	TTTGATATAA	AAAGTGAAAG	TATTAGATAT	960
	ATTTGAAAGG	TAAGTACTTC	GGTGCTTGCC	TTTTTAGGAT	1000
10	GCATATATAT	AGATTAAACC	GCACTTCTAT	ATTAATAGAA	1040
	AGTGCGGTTA	TTTATACACT	CAATCTAAAC	TATAATAATT	1080
15	GGAATCATCT	TCAA			1095

(15) INFORMATION FOR SEQUENCE ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- 20 (A) LENGTH: 266  
(B) TYPE: Amino Acid  
(C) STRANDEDNESS: Unknown  
(D) TOPOLOGY: Unknown

(ii) Molecule type: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

	Met	Asn	Lys	Ser	Arg	Phe	Ile	Ser	Cys	Val
					5					10
30	Ile	Leu	Ile	Phe	Ala	Leu	Ile	Leu	Val	Leu
					15					20
	Phe	Thr	Pro	Asn	Val	Leu	Ala	Glu	Ser	Gln
					25					30
35	Pro	Asp	Pro	Thr	Pro	Asp	Glu	Leu	His	Lys
					35					40
	Ala	Ser	Lys	Phe	Thr	Gly	Leu	Met	Glu	Asn
					45					50
	Met	Lys	Val	Leu	Tyr	Asp	Asp	His	Tyr	Val
					55					60
45	Ser	Ala	Thr	Lys	Val	Lys	Ser	Val	Asp	Lys
					65					70
	Phe	Arg	Ala	His	Asp	Leu	Ile	Tyr	Asn	Ile
					75					80
50	Ser	Asp	Lys	Lys	Leu	Lys	Asn	Tyr	Asp	Lys

					85					90	
		Val	Lys	Thr	Glu	Leu	Leu	Asn	Glu	Gly	Leu
						95					100
5		Ala	Lys	Lys	Tyr	Lys	Asp	Glu	Val	Val	Asp
						105					110
		Val	Tyr	Gly	Ser	Asn	Tyr	Tyr	Val	Asn	Cys
10						115					120
		Tyr	Phe	Ser	Ser	Lys	Asp	Asn	Val	Gly	Lys
						125					130
15		Val	Thr	Gly	Gly	Lys	Thr	Cys	Met	Tyr	Gly
						135					140
		Gly	Ile	Thr	Lys	His	Glu	Gly	Asn	His	Phe
20						145					150
		Asp	Asn	Gly	Asn	Leu	Gln	Asn	Val	Leu	Ile
						155					160
		Arg	Val	Tyr	Glu	Asn	Lys	Arg	Asn	Thr	Ile
25						165					170
		Ser	Phe	Glu	Val	Gln	Thr	Asp	Lys	Lys	Ser
						175					180
30		Val	Thr	Ala	Gln	Glu	Leu	Asp	Ile	Lys	Ala
						185					190
		Arg	Asn	Phe	Leu	Ile	Asn	Lys	Lys	Asn	Leu
35						195					200
		Tyr	Glu	Phe	Asn	Ser	Ser	Phe	Tyr	Glu	Thr
						205					210
		Gly	Tyr	Ile	Lys	Phe	Ile	Glu	Asn	Asn	Gly
40						215					220
		Asn	Thr	Phe	Trp	Tyr	Asp	Met	Met	pro	Ala
						225					230
45		Pro	Gly	Asp	Lys	Phe	Asp	Gln	Ser	Lys	Tyr
						235					240
		Leu	Met	Met	Tyr	Asn	Asp	Asn	Lys	Thr	Val
50						245					250
		Asp	Ser	Lys	Ser	Val	Lys	Ile	Glu	Val	His
						255					260

Leu Thr Thr Lys Asn Gly  
265

5 (16) INFORMATION FOR SEQUENCE ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 1837  
(B) TYPE: Nucleic Acid  
(C) STRANDEDNESS: Unknown  
(D) TOPOLOGY: Unknown

10

(ii) Molecule type: DNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

15	TCATGTTTGA CAGCTTATCA TCGATAAGCT TACTTTTCGA	40
	ATCAGGTCTA TCCTTGAAAC AGGTGCAACA TAGATTAGGG	80
	CATGGAGATT TACCAGACAA CTATGAACGT ATATACTCAC	120
20	ATCACGCAAT CGGCAATTGA TGACATTGGA ACTAAATTCA	160
	ATCAATTTGT TACTAACAAG CAACTAGATT GACAACTAAT	200
	TCTCAACAAA CGTTAATTTA ACAACATTCA AGTAACTCCC	240
25	ACCAGCTCCA TCAATGCTTA CCGTAAGTAA TCATAACTTA	280
	CTAAAACCTT GTTACATCAA GGTTTTTTCT TTTTGTCTTG	320
30	TTTCATGAGTT ACCATAACTT TCTATATTAT TGACAACTAA	360
	ATTGACAACT CTTCAATTAT TTTTCTGTCT ACTCAAAGTT	400
	TTCTTCATTT GATATAGTCT AATTCCACCA TCAC'TTCTTC	440
35	CACTCTCTCT ACCGTCACAA CTTTCATCATC TCTCACTTTT	480
	TCGTGTGGTA ACACATAATC AAATATCTTT CCGTTTTTAC	520
40	GCACTATCGC TACTGTGTCA CCTAAAATAT ACCCCTTATC	560
	AATCGCTTCT TTAAACTCAT CTATATATAA CATATTTTCAT	600
	CCTCCTACCT ATCTATTCGT AAAAAGATAA AAATAACTAT	640
45	TGTTTTTTTT GTTATTTTAT AATAAAATTA TTAATATAAG	680
	TTAATGTTTT TTAAAAATAT ACAATTTTAT TCTATTTATA	720
50	GTTAGCTATT TTTTCATTGT TAGTAATATT GGTGAATTGT	760

	AATAACCTTT TTAATCTAG AGGAGAACCC AGATATAAAA	800
	TGGAGGAATA TTAATGGAAA ACAATAAAAA AGTATTGAAG	840
5	AAAATGGTAT TTTTGTGTTT AGTGACATTT CTTGGACTAA	880
	CAATCTCGCA AGAGGTATTT GCTCAACAAG ACCCCGATCC	920
10	AAGCCAACTT CACAGATCTA GTTTAGTTAA AAACCTTCAA	960
	AATATATATT TTCTTTATGA GGGTGACCCT GTTACTCACG	1000
	AGAATGTGAA ATCTGTTGAT CAACTTAGAT CTCACGATTT	1040
15	AATATATAAT GTTTCAGGGC CAAATTATGA TAAATTAAAA	1080
	ACTGAACTTA AGAACCAAGA GATGGCAACT TTATTTAAGG	1120
20	ATAAAAACGT TGATATTTAT GGTGTAGAAT ATTACCATCT	1160
	CTGTTATTTA TGTGAAAATG CAGAAAGGAG TGCATGTATC	1200
	TACGGAGGGG TAACAAATCA TGAAGGGAAT CATTTAGAAA	1240
25	TCCTAAAAA GATAGTCGTT AAAGTATCAA TCGATGGTAT	1280
	CCAAAGCCTA TCATTTGATA TTGAAACAAA TAAAAAATG	1320
30	GTAAC TGCTC AAGAATTAGA CTATAAAGT AGAAAATATC	1360
	TTACAGATAA TAAGCAACTA TATACTAATG GACCTTCTAA	1400
	ATATGAACT GGATATATAA AGTTCATACC TAAGAATAAA	1440
35	GAAAGTTTTT GGTGTGATTT TTTCCCTGAA CCAGAATTTA	1480
	CTCAATCTAA ATATCTTATG ATATATAAAG ATAATGAAAC	1520
40	GCTTGA CTCA AACACAAGCC AAATTGAAGT CTACCTAACA	1560
	ACCAAGTAAC TTTTGTCTTT TGGCAACCTT ACCTACTGCT	1600
	GGATTTAGAA ATTTTATGTC AATTCTTTTA TTAATGTAAA	1640
45	AACCGCTCAT TTGATGAGCG GTTTGTCTT ATCTAAAGGA	1680
	GCTTTACCTC CTAATGCTGC AAAATTTTAA ATGTTGGATT	1720
50	TTTGTATTG TCTATTGTAT TTGATGGGTA ATCCCATTTT	1760
	TCGACAGACA TCGTCGTGCC ACCTCTAACA CCAAAATCAT	1800

AGACAGGAGC TTGTAGCTTA GCAACTATTT TATCGTC

1837

## (17) INFORMATION FOR SEQUENCE ID NO:16:

5 (i) SEQUENCE CHARACTERISTICS:  
 (A) LENGTH: 251  
 (B) TYPE: Amino Acid  
 (C) STRANDEDNESS: Unknown  
 (D) TOPOLOGY: Unknown

10 (ii) Molecule type: Peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

15	Met Glu Asn Asn Lys Lys Val Leu Lys Lys	5	10
	Met Val Phe Phe Val Leu Val thr Phe Leu	15	20
20	Gly Leu Thr Ile Ser Gln Glu Val Phe Ala	25	30
	Gln Gln Asp Pro Asp Pro Ser Gln Leu His	35	40
25	Arg Ser Ser Leu Val Lys Asn Leu Gln Asn	45	50
	Ile Tyr Phe Leu Tyr Glu Gly Asp Pro Val	55	60
30	Thr His Glu Asn Val Lys Ser Val Asp Gln	65	70
	Leu Arg Ser His Asp Leu Ile Tyr Asn Val	75	80
35	Ser Gly Pro Asn Tyr Asp Lys Leu Lys Thr	85	90
40	Glu Leu Lys Asn Gln Glu Met Ala Thr Leu	95	100
	Phe Lys Asp Lys Asn Val Asp Ile Tyr Gly	105	110
45	Val Glu Tyr Tyr His Leu Cys Tyr Leu Cys	115	120
50	Glu Asn Ala Glu Arg Ser Ala Cys Ile Tyr	125	130



	Gly Gly Val Thr Asn His Glu Gly Asn His	135	140
5	Leu Glu Ile Pro Lys Lys Ile Val Val Lys	145	150
	Val Ser Ile Asp Gly Ile Gln Ser Leu Ser	155	160
10	Phe Asp Ile Glu Thr Asn Lys Lys Met Val	165	170
	Thr Ala Gln Glu Leu Asp Tyr Lys Val Arg	175	180
15	Lys Tyr Leu Thr Asp Asn Lys Gln Leu Tyr	185	190
20	Thr Asn Gly Pro Ser Lys Tyr Glu Thr Gly	195	200
	Tyr Ile Lys Phe Ile Pro Lys Asn Lys Glu	205	210
25	Ser Phe Trp Phe Asp Phe Phe Pro Glu Pro	215	220
	Glu Phe Thr Gln Ser Lys Tyr Leu Met Ile	225	230
30	Tyr Lys Asp Asn Glu Thr Leu Asp Ser Asn	235	240
35	Thr Ser Gln Ile Glu Val Tyr Leu Thr Thr	245	250
	Lys		
40			
45			

What is claimed is:

1. An isolated and purified superantigen toxin DNA fragment which has been altered such that  
5 binding of the encoded altered toxin to either the MHC class II or T cell antigen receptor is altered.
2. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin  
10 is Staphylococcal enterotoxin A having the sequence of SEQ ID NO:1 or a portion thereof, or an allelic portion thereof.
3. An isolated and purified DNA fragment  
15 according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin A having the sequence of SEQ ID NO:3 or a portion thereof, or an allelic portion thereof.
- 20 4. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin B having the sequence of SEQ ID NO:5 or a portion thereof, or an allelic  
25 portion thereof.
5. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin B having the sequence of  
30 SEQ ID NO:7 or a portion thereof, or an allelic portion thereof.
6. An isolated and purified DNA fragment according to claim 1, wherein said superantigen toxin is Staphylococcal enterotoxin B having the sequence of

SEQ ID NO:9 or a portion thereof, or an allelic portion thereof.

7. An isolated and purified DNA fragment  
5 according to claim 2, wherein said fragment encodes the amino acid sequence of SEQ ID NO:2 or a portion thereof, or an allelic portion thereof.

8. An isolated and purified DNA fragment  
10 according to claim 3, wherein said fragment encodes the amino acid sequence of SEQ ID NO:4 or a portion thereof, or an allelic portion thereof.

9. An isolated and purified DNA fragment  
15 according to claim 4, wherein said fragment encodes the amino acid sequence of SEQ ID NO:6 or a portion thereof, or an allelic portion thereof.

10. An isolated and purified DNA fragment  
20 according to claim 5, wherein said fragment encodes the amino acid sequence of SEQ ID NO:8 or a portion thereof, or an allelic portion thereof.

11. An isolated and purified DNA fragment  
25 according to claim 6, wherein said fragment encodes the amino acid sequence of SEQ ID NO:10 or a portion thereof, or an allelic portion thereof.

12. A recombinant DNA construct comprising:  
30 (i) a vector, and  
(ii) an isolated and purified altered superantigen toxin DNA fragment according to claim 1.

13. A recombinant DNA construct according to  
35 claim 12, wherein said DNA fragment has the sequence

according to SEQ ID NO:1 or a portion thereof, or an allelic portion thereof.

14. A recombinant DNA construct according to  
5 claim 12, wherein said DNA fragment has the sequence according to SEQ ID NO:3 or a portion thereof, or an allelic portion thereof.

15. A recombinant DNA construct according to  
10 claim 12, wherein said DNA fragment has the sequence according to SEQ ID NO:5 or a portion thereof, or an allelic portion thereof.

16. A recombinant DNA construct according to  
15 claim 12, wherein said DNA fragment has the sequence according to SEQ ID NO:7 or a portion thereof, or an allelic portion thereof.

17. A recombinant DNA construct according to  
20 claim 12, wherein said DNA fragment has the sequence according to SEQ ID NO:9 or a portion thereof, or an allelic portion thereof.

18. The recombinant DNA construct according  
25 to claim 13, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:2.

19. The recombinant DNA construct according  
to claim 14, wherein said DNA fragment encodes the  
30 amino acids sequence specified in SEQ ID NO:4.

20. The recombinant DNA construct according  
to claim 15, wherein said DNA fragment encodes the  
amino acids sequence specified in SEQ ID NO:6.

21. The recombinant DNA construct according to claim 16, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:8.

5

22. The recombinant DNA construct according to claim 17, wherein said DNA fragment encodes the amino acids sequence specified in SEQ ID NO:10.

10

23. A recombinant DNA construct according to claim 13 wherein said construct is pETA489270P.

24. A recombinant DNA construct according to claim 14 wherein said construct is pETA489270C.

15

25. A recombinant DNA construct according to claim 15 wherein said construct is pETB2360210.

26. A recombinant DNA construct according to claim 16 wherein said construct pETB899445P.

20

27. A recombinant DNA construct according to claim 17 wherein said construct is pETB899445C.

28. A recombinant DNA construct according to claim 12, wherein said vector is an expression vector.

25

29. A host cell transformed with a recombinant DNA construct according to claim 12.

30

30. A host cell transformed with a recombinant DNA construct according to claim 18.

31. A host cell transformed with a recombinant DNA construct according to claim 19.

35

32. A host cell transformed with a recombinant DNA construct according to claim 20.

5 33. A host cell transformed with a recombinant DNA construct according to claim 21.

34. A host cell transformed with a recombinant DNA construct according to claim 22.  
10

35. A host cell according to claim 29, wherein said cell is prokaryotic.

36. A host cell according to claim 30,  
15 wherein said cell is prokaryotic.

37. A host cell according to claim 31, wherein said cell is prokaryotic.

20 38. A host cell according to claim 32, wherein said cell is prokaryotic.

39. A host cell according to claim 33, wherein said cell is prokaryotic.  
25

40. A host cell according to claim 34, wherein said cell is prokaryotic.

41. A method for producing altered  
30 superantigen toxin comprising culturing the cells according to claim 29, under conditions such that said DNA fragment is expressed and said superantigen toxin is thereby produced, and isolating said superantigen toxin.

42. A method for producing altered  
superantigen toxin comprising culturing the cells  
according to claim 30, under conditions such that said  
5 DNA fragment is expressed and said superantigen toxin  
is thereby produced, and isolating said superantigen  
toxin.

43. A method for producing altered  
10 superantigen toxin comprising culturing the cells  
according to claim 31, under conditions such that said  
DNA fragment is expressed and said superantigen toxin  
is thereby produced, and isolating said superantigen  
toxin.

15 44. A method for producing altered  
superantigen toxin comprising culturing the cells  
according to claim 32, under conditions such that said  
DNA fragment is expressed and said superantigen toxin  
20 is thereby produced, and isolating said superantigen  
toxin.

45. A method for producing altered  
superantigen toxin comprising culturing the cells  
25 according to claim 33, under conditions such that said  
DNA fragment is expressed and said superantigen toxin  
is thereby produced, and isolating said superantigen  
toxin.

30 46. A method for producing altered  
superantigen toxin comprising culturing the cells  
according to claim 34, under conditions such that said  
DNA fragment is expressed and said superantigen toxin  
is thereby produced, and isolating said superantigen  
35 toxin.

47. An isolated and purified superantigen toxin which has been altered such that binding of the encoded altered toxin to either the MHC class II or T cell antigen receptor is altered.

48. An isolated and purified superantigen toxin according to claim 47 wherein said toxin is staphylococcal enterotoxin A.

10

49. An isolated and purified superantigen toxin according to claim 47 wherein said toxin is staphylococcal enterotoxin B.

15

50. An altered SEA superantigen toxin peptide according to claim 48 wherein position 92 has been changed to alanine.

51. An altered SEA superantigen toxin peptide according to claim 48 wherein position 70 has been changed to arginine.

20

52. An altered SEA superantigen toxin peptide according to claim 48 wherein position 48 has been changed to arginine.

25

53. An altered SEA superantigen toxin peptide according to claim 48 wherein position 64 has been mutated to alanine.

30

54. An altered SEB superantigen toxin peptide according to claim 49 wherein position 115 has been changed to alanine.



55. An altered SEB superantigen toxin peptide according to claim 49 wherein position 89 has been changed to alanine.

5 56. An altered SEB superantigen toxin peptide according to claim 49 wherein position 67 has been changed to glutamine.

57. An altered SEB superantigen toxin  
10 peptide according to claim 49 wherein position 94 has been changed to alanine.

58. An altered SEB superantigen toxin peptide according to claim 49 wherein position 61 has  
15 been changed to alanine.

59. A method for the diagnosis of superantigen-associated bacterial infection comprising the steps of:

20 (i) contacting a sample from an individual suspected of having a superantigen-associated bacterial infection with altered superantigen toxin; and

(ii) detecting the presence or absence of a  
25 superantigen-associated bacterial infection by detecting the presence or absence of a complex formed between the altered superantigen toxin and antibodies specific therefor in the sample.

30 60. A method for the diagnosis of a superantigen toxin-associated bacterial infection according to claim 42 wherein the altered superantigen toxin is chosen from the group consisting of SEB, and SEA.

61. A superantigen toxin-associated infection diagnostic kit comprising an altered superantigen toxin according to claim 1 wherein said  
5 toxin is chosen from the group consisting of SEB, and SEA, and ancillary reagents suitable for use in detecting the presence or absence of antibodies against superantigen toxin in a mammalian sample.

10 62. A vaccine comprising an altered superantigen toxin according to claim 1 effective for the production of antigenic and immunogenic response resulting in the protection of a mammal against superantigen-associated bacterial infection.

15 63. A vaccine according to claim 62 wherein said altered superantigen toxin is chosen from the group consisting of SEB, and SEA.

20 64. A vaccine according to claim 63 wherein said vaccine further comprises at least one other different altered superantigen toxin chosen from the group consisting of SEB, and SEA.

25 65. A vaccine according to claim 62, wherein the superantigen toxin is SEB and the vaccine is identified as B899445.

30 66. A vaccine according to claim 62, wherein the superantigen toxin is SEA and the vaccine is identified as A489270.

35 67. A bivalent vaccine according to claim 64 wherein said altered superantigen toxins are SEA and SEB.

68. A bivalent vaccine according to claim 67 wherein said toxin SEA is A489270 and SEB is B899445.

5           69. A multivalent vaccine against superantigen-associated bacterial infections comprising a combination of altered superantigen toxins selected from the group consisting essentially of SEA, and SEB, or any portion or allelic form  
10 thereof, capable of eliciting protective antibodies against superantigen toxins in a pharmaceutically acceptable excipient in a pharmaceutically acceptable amount.

15           70. A therapeutic method for the treatment or amelioration of a superantigen-associated bacterial infection said method comprising administering to an individual in need of such treatment an effective  
20 amount of sera from individuals immunized with one of more altered superantigen toxin vaccine according to claim 62 in a pharmaceutically acceptable dose in a pharmaceutically acceptable excipient.

25           71. A therapeutic method for the treatment or amelioration of a superantigen-associated bacterial infection, said method comprising administering to an individual in need of such treatment an effective  
30 amount of antibodies against altered superantigen toxins in a pharmaceutically acceptable dose in a pharmaceutically acceptable excipient.

          72. A therapeutic method for the treatment or amelioration of a superantigen-associated bacterial infection, said method comprising administering to an  
35 individual in need of such treatment an effective

amount of altered superantigen toxins from  
staphylococcal bacteria in order to inhibit adhesion  
of superantigen bacterial toxin to MHC class II or T  
cell receptors by competitive inhibition of these  
5 interactions in a pharmaceutically acceptable dose in  
a pharmaceutically acceptable excipient.

73. A therapeutic method for the treatment  
of diseases that may not be associated directly with  
10 superantigen toxins by causing specific  
nonresponsiveness of T cell subsets or by expanding or  
stimulating specific T cell subsets, in vivo or ex  
vivo by use of altered superantigen toxin.

15

20

25

30

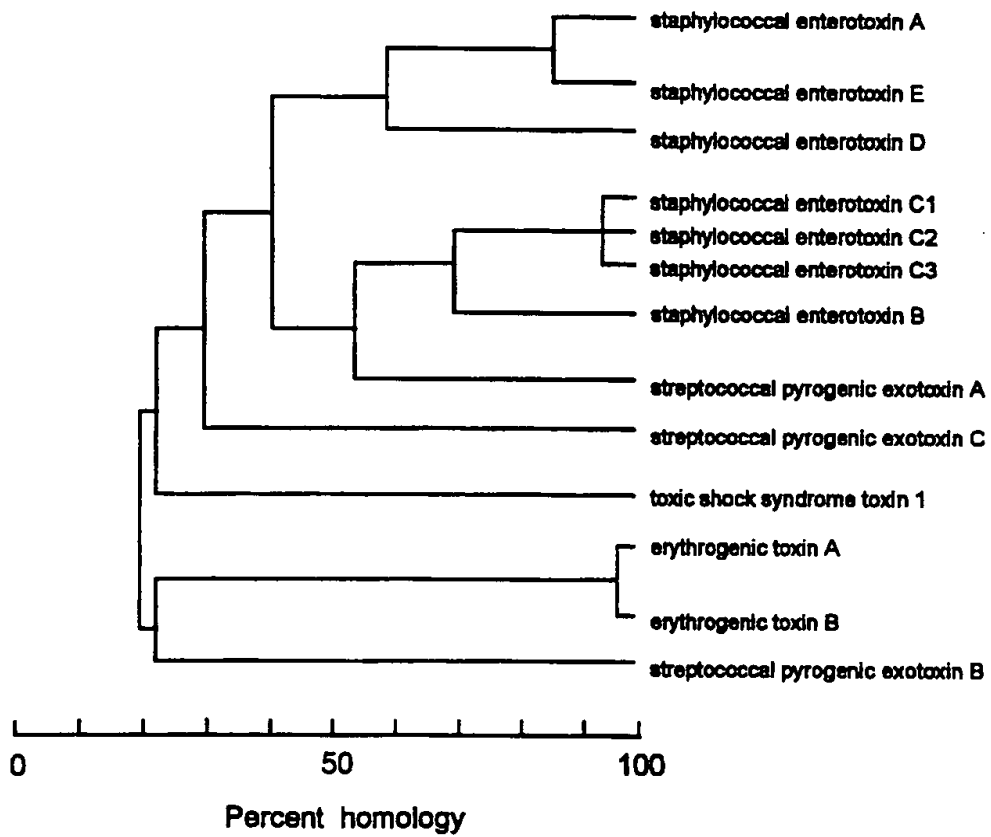


FIGURE 1

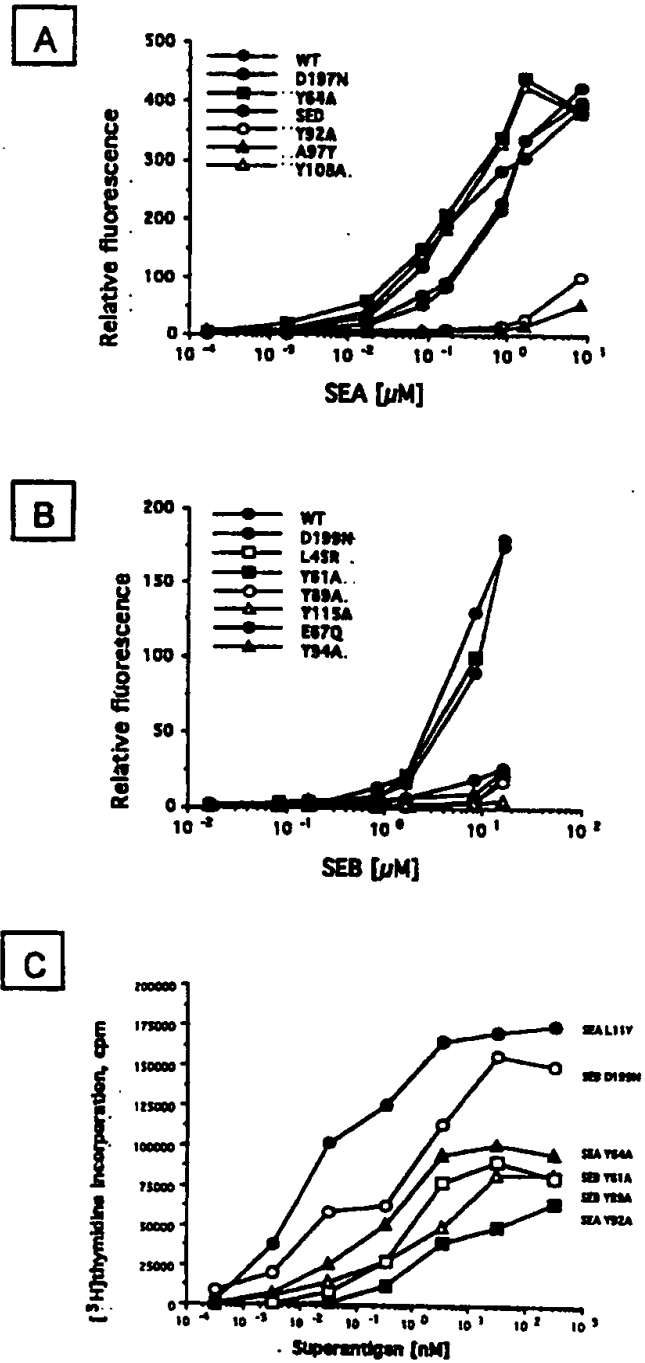


Fig. 2

SEA	...	SHDQF	QHTILFKGFFTDHISWYNDLLV	70	FDSKDIVDKYK.GKKVDLYGAY	92	GYQCA.....GGTPNKTACM	108	GGVTLHDNNRLTEKK
SED	...	TGDQF	ENTLLYKKFFTDLINFEDELLI		FNSKEMAQHPK.SKNVDVYPIR		SINCY.....GGEIDRTACT		GGVTPHEGNKLERKK
SEE	...	SDDQF	ENTLLFKGFFTGHPWYNDLLV		LGSKDATNKKYK.GKKVDLYGAY		GYQCA.....GGTPNKTACM		GGVTLHDNNRLTEKK
SEB	...	SIDQF	YFDLIYSIKDTKLGNYDNVRV		FNKOLADKYK.DKYVDVPGAN		YYQCYFSSKKTNDINSHQTDKRT		CM.GGVTEHNGQLD..KY
SEC1	...	SVDKF	AHDLIYNI SDKLLKNDYDKVKT		LLNEGLAKKYK.DEWVDVYGSN		YVNCYFSSKKNVGVKVTGG...KT.CM		GGITKHEGNHFDNGNL
SEC2	...	SVDKF	AHDLIYNI SDKLLKNDYDKVKT		LLNEGLAKKYK.DEWVDVYGSN		YVNCYFSSKKNVGVKVTGG...KT.CM		GGITKHEGNHFDNGNL
SEC3	...	SVDKF	AHDLIYNI SDKLLKNDYDKVKT		LLNEGLAKKYK.DEWVDVYGSN		YVNCYFSSKKNVGVKVTGG...KT.CM		GGITKHEGNHFDNGNL
SPea	...	SVDQL	SHDLIYNVSG...PNYDKLKT		LNQEMATLPK.DKNVDIYGV		YHLCYLCEAAE.....RSACI		GGVTNHEGNHLEIPK.
TSST1	...	VLDNS	GSMRIKNTD.....GSISLI		FPSPYYSPAFTKGEKVDLNTKR		KKSQHTSEG.....TYIHF.Q		SGVTNT EKLPT...P

Fig. 3

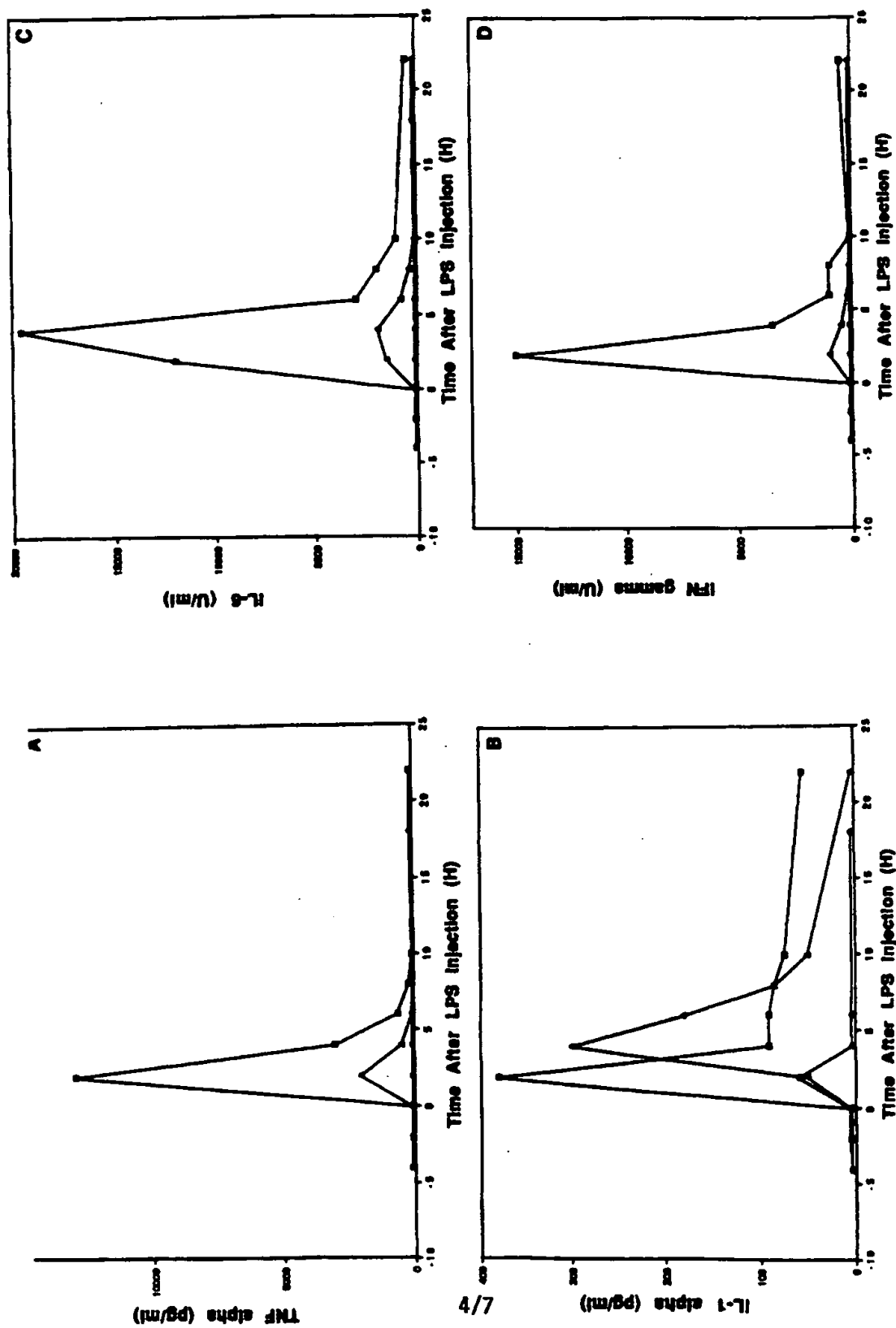


Fig. 4



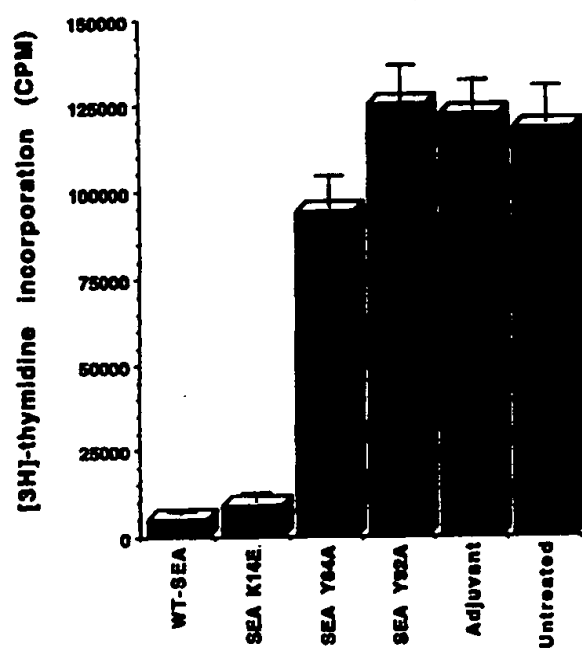


Fig. 5

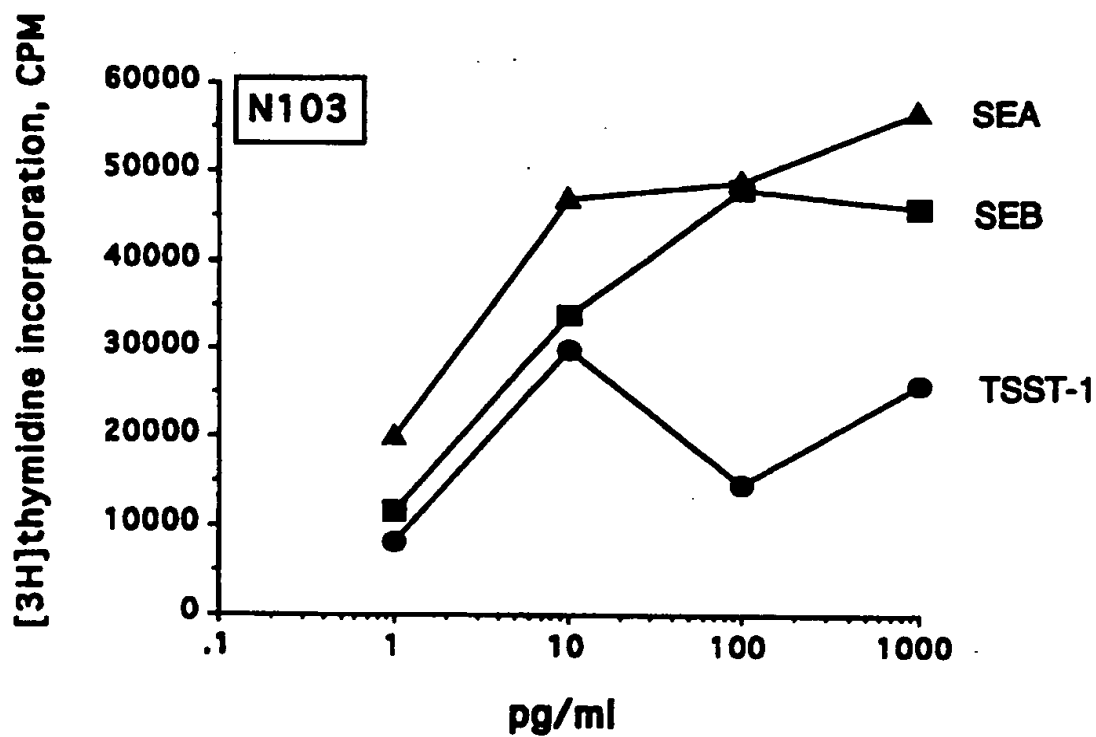
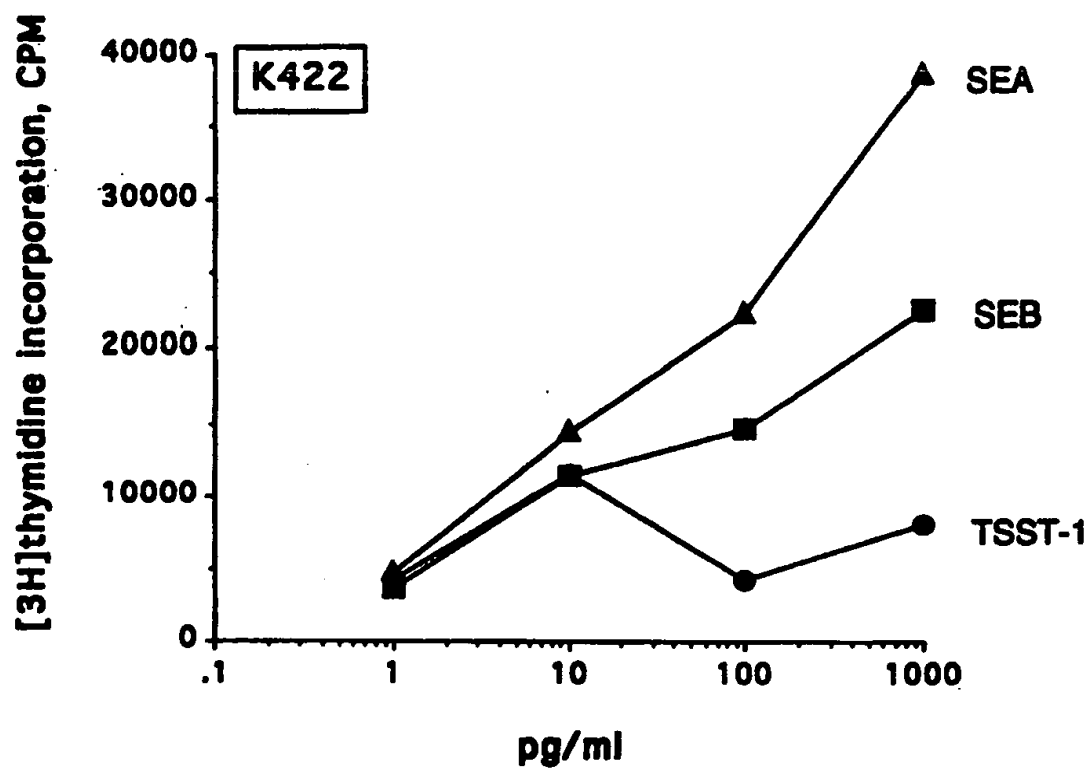


Fig. 6

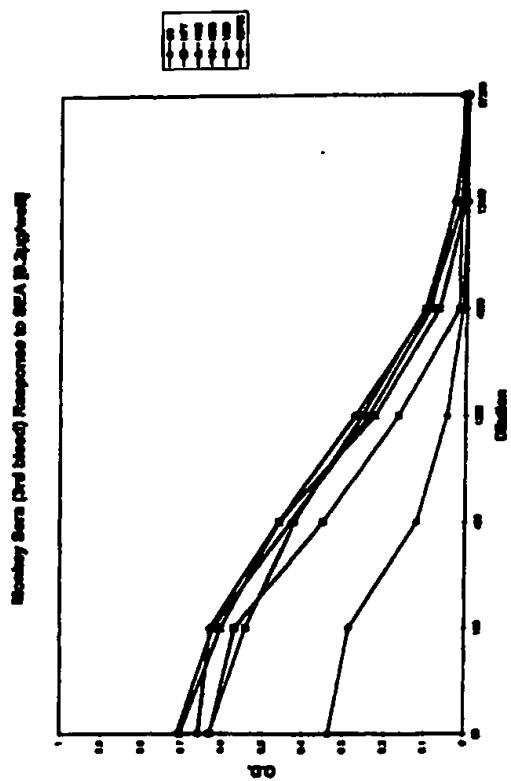
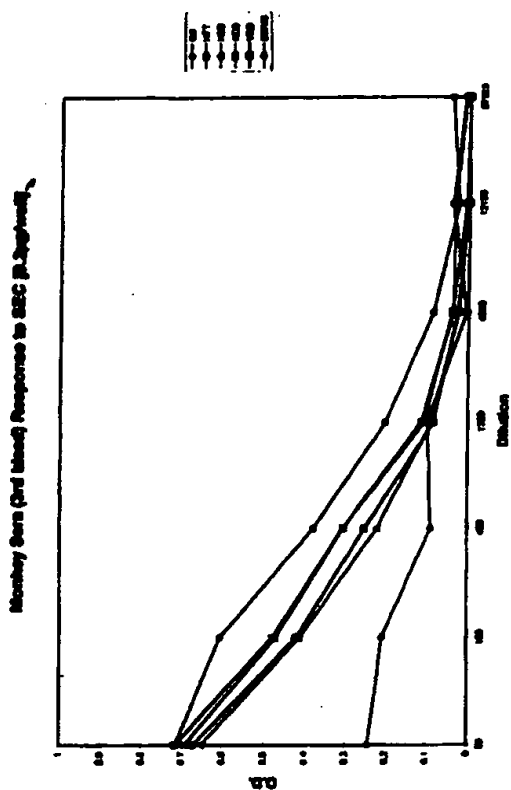
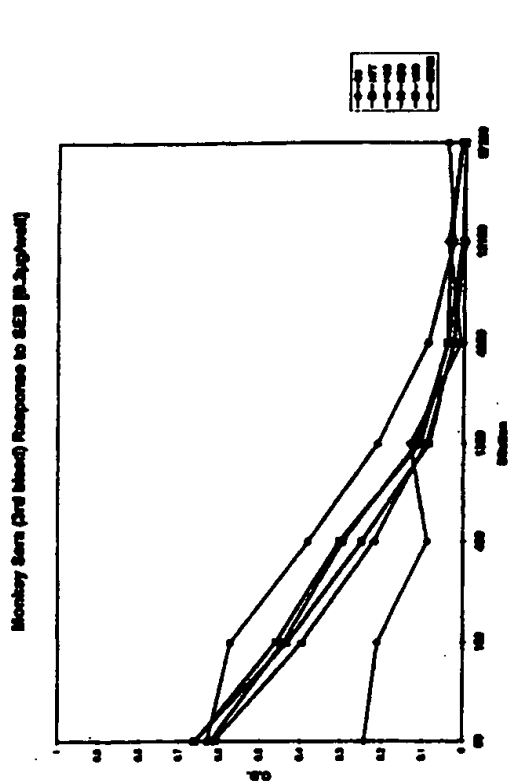


Fig 7

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/16766

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : Please See Extra Sheet

US CL : Please See Extra Sheet

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/184.1, 234.1, 236.1, 237.1, 243.1; 435/320.1, 252.3, 172.3, 69.1, 69.3, 70.1, 71.1, 7.1; 530/350+; 536/23.1; 436/500

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	DANNECKER et al. Activation of human T cells by the superantigen Staphylococcus enterotoxin B: Analysis on a cellular level. Immunobiology. 1994, Vol. 190, pages 116-126, see entire document.	1-73
A	GONZALO et al. Expansion and clonal deletion of peripheral T cells induced by bacterial superantigen is independent of the interleukin-2 pathway. Eur. J. Immunol. 1992, Vol. 22, pages 1007-1011, see entire document.	1-73
Y	BAVARI et al. Genetically attenuated bacterial superantigen vaccines. J. Cellular Biochemistry. 1995, Suppl. 21A, abstract C2-204, page 88.	1-73

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:	*T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
*A* document defining the general state of the art which is not considered to be of particular relevance	*X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
*B* earlier document published on or after the international filing date	*Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
*L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	*A* document member of the same patent family
*O* document referring to an oral disclosure, use, exhibition or other means	
*P* document published prior to the international filing date but later than the priority date claimed	

Date of the actual completion of the international search

08 NOVEMBER 1998

Date of mailing of the international search report

17 DEC 1998

Name and mailing address of the ISA/US  
Commissioner of Patents and Trademarks  
Box PCT  
Washington, D.C. 20231

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NITA M. MINNIFIELD

Telephone No. (703) 308-0196

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/16766

## C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BAVARI et al. Staphylococcal Enterotoxin A and toxic shock syndrome toxin compete with CD4 for human major histocompatibility complex class II binding. Infection and Immunity. February 1995, Vol. 63, No. 2, pages 423-429, see entire document.	1-73
A	MAHANA et al. A natural mutation of the amino acid residue at position 60 destroys staphylococcal enterotoxin A murine T-cell mitogenicity. Infection and Immunity. August 1995, Vol. 63, No. 8, pages 2826-2832, see entire document.	1-73
A	ULRICH et al. Bacterial superantigens in human disease: structure, function and diversity. Trends in Microbiology. 1995, Vol. 3, No. 12, pages 463-468, see entire document.	1-73
A	WOODY et al. Differential immune responses to Staphylococcal enterotoxin B mutations in a hydrophobic loop dominating the interface with major histocompatibility complex class II receptors. J. Infectious Diseases. 1998, Vol. 177, pages 1013-1022, see entire document.	1-73
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X		62-69

# INTERNATIONAL SEARCH REPORT

International application No.  
PCT/US98/16766

## A. CLASSIFICATION OF SUBJECT MATTER: IPC (6):

A61K 39/00, 39/44, 39/02, 39/085; C12N 15/63+, 1/20, 1/21, 15/00; C12P 21/00+; G01N 33/53; C07K 14/00+

## A. CLASSIFICATION OF SUBJECT MATTER: US CL :

424/184.1, 234.1, 236.1, 237.1, 243.1; 435/320.1, 252.3, 172.3, 69.1, 69.3, 70.1, 71.1, 7.1; 530/350+, 536/23.1; 436/500

## B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, MEDLINE, BIOSIS, JAPIO, WPIDS, CABA, AGRICOLA, BIOTECHDS, CAPLUS, LIFESCI, CONFSCI, DISSABS, EMBASE

search terms: inventor names, enterotoxin A, enterotoxin B, bacterial superantigen, vaccine, dna, test, diagnosis, treatment, sea, seb, bivalent vaccine, staphylococcus, t-cell anergy, infection, mutant, fusion